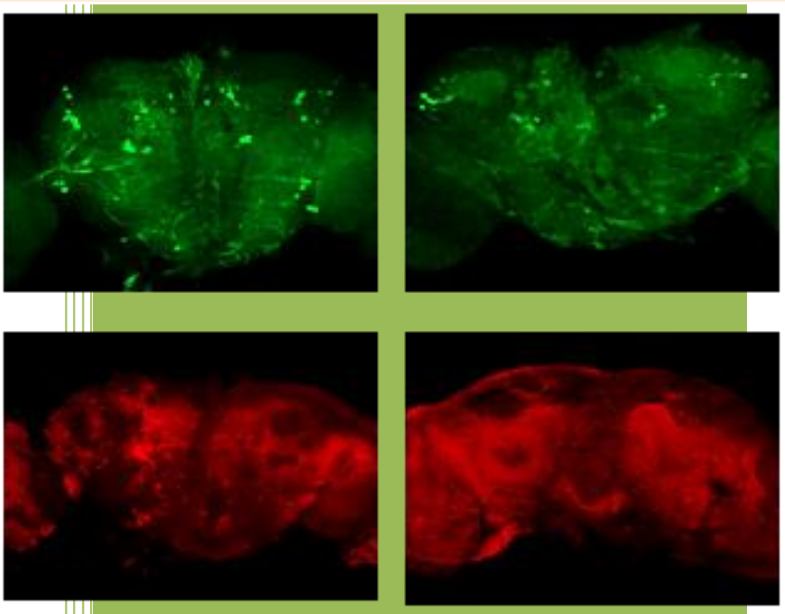
PRESIDENCY UNIVERSITY
KOLKATA

Understanding the regulation of mitochondrial homeostasis by SARM1 and its role in neurodegeneration



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The Convener

Departmental PhD committee

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Kolkata

Sub: Submission of "5000 words" summary of my PhD thesis entitled "Understanding the regulation of mitochondrial homeostasis by SARM1 and its role in neurodegeneration"

Respected Sir,

I, Ms. Malinki Sur, am a research scholar of Dept. of Life Sciences (Enrolment no.: SC/DOC/13-14/30; Registration No.: R-14RS18210030) would like to submit my "5000 words" summary of my PhD thesis entitled **"Understanding the regulation of mitochondrial homeostasis by SARM1 and its role in neurodegeneration"**.

I shall be highly obliged if you kindly consider my submission and allow me to defend my research work before the committee. The required documents are attached herewith for your kind perusal.

Thanking you,

Ms. Malinki Sur

Enrolment no.: SC/DOC/13-14/30

Dept. of Life Sciences

Presidency University

Kolkata

Enclosures:

1. 4 copies of synopsis
2. Front page of published papers
3. Certificates of attended seminars

Synopsis (5000 words)

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Title of the Thesis: **"Understanding the regulation of mitochondrial homeostasis by SARM1 and its role in neurodegeneration"**

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Title: Understanding the regulation of mitochondrial homeostasis by SARM1 and its role in neurodegeneration

1. Introduction

Neurons are the structural and functional units of the nervous system [1]. With the increase in life expectancy neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) are on rise and involve slow and progressive loss of neuron structure and function. While neurodegeneration has been studied in the context of various pathological conditions, not much is known on the molecular mechanisms that cause/regulate axonal retraction during age-related neurodegeneration. Identification of key targets that delay this process of axonal retraction before they reach the 'point of no return' may enhance axonal longevity and prevent subsequent neurodegeneration.

SARM1 (Sterile alpha and TIR motif-containing 1) is a key molecule that plays a pivotal role in axonal death [2]. The evidence came from Sarm1$^{-/-}$ flies identified through forward genetic screen where the neurons were protected from injury induced axonal death[3]. SARM1 mediated programmed axonal degeneration involves dimerization of its TIR domain and activation of its intrinsic NADase activity [4–6] The reduced NAD^+ levels induced by SARM1 causes severe energy depletion within the cells and subsequent loss of neuron[7]. It has been previously shown that SARM1 is required for mitochondrial reactive oxygen species (ROS) generation and mitochondrial damage in virus-induced neuronal death [8]. However, the detailed mechanism of SARM1 mediated mitochondrial dysfunction and how it regulates neuronal homeostasis is not well understood. *The goal of this study is to provide a detailed mechanistic insight into the regulation of neuronal homeostasis by SARM1 and its effect on the neurodegenerative pathway that may provide a common route of therapeutics in the treatment of several neurodegenerative diseases.*

2. Review of Literature

Mitochondrial defects are quite common in several models of axonal degeneration and aging and provide a significant point of convergence of metabolic regulators that maintain cellular homeostasis[9,10]. Bioenergetic changes in the neurons

associated with defective respiration in the axonal mitochondria are emerging as important phenomenon in the etiology of neurodegenerative diseases [11,12].

Previous studies have implicated the important role of the TLR adaptor protein SARM1 in mitochondrial dysfunction and axonal degeneration ultimately leading to neuronal death and hence SARM1 seem to be an attractive candidate in neurodegeneration[2,13]. However, the mechanism of SARM1 mediated mitochondrial dysfunction has not been well established yet. It has been shown that influx of extracellular calcium is necessary to induce Wallerian degeneration. It has been recently shown that SARM1 induced axon degeneration could be reversed in the presence of NAD^+ precursors[14]. Nicotinamide riboside (NR) is a NAD^+ precursor which is converted to nicotinamide mononucleotide (NMN) by the enzyme NAMPT and ultimately to NAD+ by the enzyme NMNAT. NAD+ acts as a substrate for nuclear enzymes, such as SIRT1 and PARP1 and helps in neuronal survival [15]. NAD+ is also implicated in several cellular processes like autophagy/mitophagy to prevent the toxic accumulation of misfolded proteins or damaged organelles[16]. Impaired mitophagy may lead to the accumulation of damaged mitochondria that results in excess ROS generation [17,18], which may cause further damage to the neighbouring mitochondria and subsequent axon loss [19]. How Sarm1 plays a role in mitochondrial homeostasis and regulates intracellular ROS levels has not been established yet.

Although both SARM1 and NAD^+ has been shown to regulate neuronal homeostasis, no direct evidence is available to show that NAD^+ loss due to SARM1 is responsible for such age-related neurodegeneration. However, due to the difficulty in establishing a good model for aging studies there have been limitations in getting a comprehensive understanding what regulates neuronal survival in aging and how NAD^+ may play an important role in the process.

Environmental risk factors like pesticide exposure have been implicated in the increased incidence of age-related neurodegenerative diseases like Parkinson's disease (PD) but the etiology remains unknown. There is also lack of a proper animal model system to study the progressive effect of these environmental toxins on age-associated neurodegeneration. It has been shown that chronic low dose exposure to the mitochondrial complex I inhibitor rotenone in Drosophila increases the locomotor impairment and results in dopaminergic neuronal loss [20]. However, these studies failed to correlate the increased inflammatory response with the motor deficits or dopaminergic neuron loss as seen in PD. This study proposes to analyze the

regulatory role of the neurodegenerative molecule SARM1 in regulating neuronal death in both neuronal cell lines as well as the model organism *Drosophila melanogaster* exposed to the mitochondrial complex I inhibitor rotenone, a pesticide implicated in sporadic cases of PD.

3. Objectives

i. To understand the biochemical mechanisms of the role of SARM1 in mitochondrial electron transport chain (ETC) activity.

ii. To determine the site of ROS generation by analyzing the formation of respiratory complexes

iii. To investigate the effect of excess ROS generation on mitochondrial homeostasis and susceptibility to environmental stress in aging

4. Materials and Methods

4.1 Plasmid constructs

Standard PCR and cloning techniques were used to generate the various SARM1 truncations in pCMV6 vector (Table 1). Full-length expression constructs; C-terminal Flag-tagged SARM1 was used as a template, which was a kind gift from Karin E. Peterson (Rocky Mountain Laboratories, Hamilton, USA). All expression constructs were sequenced to ensure that the fusion was in the correct reading frame and there were no additional mutations.

4.2 Cell Culture

Human SH-SY5Y cells were procured from the cell repository at NCCS, Pune. SH-SY5Y cells were grown in in DMEM-F12 medium (Gibco,USA), containing 10% FBS (Gibco, USA) and 1% Penicillin streptomycin (Gibco, USA) at 37^{o}C and 5% CO_2 in a humidified chamber. Cells were sub cultured at 90-95% confluency.

4.3 Treatments

Rotenone (Sigma, USA), FCCP (Abcam, USA), Oligomycin (Sigma), Nimodipine (Abcam), Bafilomycin (Sigma) and Resveratrol (Sigma) were freshly prepared as 2 mM, 1 mM, 1 mM, 10 mM, 10mM and 10mM stock solutions respectively in DMSO (Sigma, USA). Antimycin (Sigma) was prepared freshly as 5mM stock in Ethanol

(Himedia) and sodium arsenite (Sigma), N-Acetyl Cysteine (Sigma), Ncotinamide riboside (ChromaDex, USA), EGTA (SRL, India) and Chloroquine were prepared freshly as 1 mM, 100 mM, 10 mM, 10 mM and 10mM stock respectively in water before the start of the experiment, and subsequently dilutions were made in cell culture medium.

4.4 Cell viability Assay

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to estimate the viability of SH-SY5Y cells. MTT was prepared freshly as 5 mg/ml stock in 1X PBS (Himedia). SH-SY5Y cells were cultured in 24-well plates. At specific time points, triplicate wells per treatment were incubated with MTT reagent at a final concentration of 0.5 mg/ml for 3 hours to allow production of purple formazan. The MTT solution was aspirated and the cells were lysed in DMSO. The formazan concentration in each well was measured by taking absorbance at 540 nm using a cell plate reader (Synergy, Bio Tek). Treated sample data were compared with control samples to determine percentage cell death.

4.5 Reactive Oxygen Species generation assay

Cells: Dichloroflouricinediacetate (DCFDA, Sigma) was freshly prepared as 1mM stock in ethanol. SH-SY5Y cells were incubated with DCFDA at a final concentration of 50µM for 30min at 37ºC. Cells were washed and resuspended in fresh media and fluorescence was measured by using a cell plate reader at 485 nm excitation/530 nm emission.

Flies: To assess the levels of ROS, 1-day old flies were grown in rotenone containing diet in the presence of absence of NAC. To measure ROS levels, heads of 10-day old flies treated as described were homogenized in extraction buffer containing 50 µM of 2,7-dichlorofluorescein diacetate (DCFDA) and incubated at room temperature for 60 min. Following incubation fluorescence was measured at 485 nm excitation/530 nm emission.

4.6 Mitochondrial superoxide

MitoSOX Red (Invitrogen, USA), mitochondrial superoxide indicator was used to detect superoxide in mitochondria. MitoSOX stock of 5 mM was made in DMSO. SH-SY5Y cells were cultured in 8-well chamber slides and incubated with Hank's balanced

salt solution (Thermo Scientific, USA) at a final concentration of 5 µM for 10 min. The relative intensity (510 nm excitation/580 nm emission) of mitochondrial superoxide generation was analysed using a fluorescence microscope (Leica).

4.7 Nuclear Staining

4',6-Diamidino-2-phenylindole dihydrochloride (DAPI; Biorad Laboratories, USA) was used to stain nucleus. 100X DAPI was prepared in methanol and stored at -20 ˚C at dark. SH-SY5Y cells were cultured in 8-well chamber slides and incubated with staining solution at a final concentration of 1X for 15 min after fixing cells with 2% formaldehyde (Sigma, cat no: 47608). Cells were analysed by fluorescence microscope (359 nm excitation/461 nm emission).

4.8 Immunocytochemistry

SH-SY5Y cells were cultured in 8-well chamber slides. Cells were fixed with 2% formaldehyde and permeabilized by the treatment with PBST (0.1% Triton X-100; Amresco, cat no: 694 in PBS) for 5 min at 4-degree C. After blocking for 1h, cells were incubated with respective first antibodies, washed twice and the samples were incubated with Alexa Fluor 488 and 594 goat anti-mouse (ab150105) and rabbit IgG antibody (ab150074). Then cells were labelled with DAPI in PBS for 5 min, washed for 10 min in PBS and mounted in Fluoroshield mounting media (Abcam) and images were taken under fluorescence microscope.

4.9 Transfection

SH-SY5Y cells were transiently transfected with genes of interest (Full length SARM1 and different deletion mutants) using lipofectamine 2000 (Thermoscientific). 2.5 ug of DNA was used to transfect 35 mm plate with 2.5 µl of lipofectamine. For siRNA transfection assays, SH-SY5Y cells were grown for 24 h and transfected with 25 nM of Sarm1 siRNA (Ambion, Life Technologies), TNFα siRNA (Ambion, Life Technologies) or with a nontargeting siRNA (Ambion, Life Technologies) as a control using Lipofectamine 2000 for 48 h and following transfection cells were treated with 5 µM of rotenone for 24 h. For TNFα blocking studies, SH-SY5Y cells were cultured and pre-treated with TNFα neutralizing antibody (Abcam) at a final concentration of 5 mg/ml for 1 h to neutralize TNF α cytotoxicity activity in vitro. Cells were then treated with 5 µM of rotenone for 24 h.

4.10 Fly strains used

Wild-type w^{1118} strains of fly were generously obtained from Dr. Rupak Datta, IISER, Kolkata. Both male and female flies were grown at 22 °C and 12-h light/dark cycle. Flies were fed on standard cornmeal/molasses diet and changed every 3–5 days. Only male flies were used in this study, since, female flies show altered physiological condition because of the reproductive development.

4.11 Treatment

Cohorts of twenty flies of both genders per vials were placed on the rotenone containing diet at 1-day, 10-day, and 20-day post-eclosion. Rotenone was added to the fly food to achieve final concentrations of 50, 100, and 200 μM. Flies were transferred to fresh rotenone containing medium every 2 days and were monitored for survival and locomotor activity as described below. For aging studies, flies were grown for either 10 or 20-days in regular medium following eclosion. At day-10 or day-20 they were fed in rotenone containing diet and experiments were performed as described. For a few experiments flies were exposed to either rotenone or sodium arsenite in the presence or absence of 1 mg/ml of N-acetyl-L-cysteine (NAC).

4.12 Fly survival and negative geotaxis assay

Flies were chronically exposed to 50, 100, and 200 μM of rotenone 1-day, 10-day, and 20-days post-eclosion. For each condition, at least five vials containing twenty flies of both genders were raised on diet with or without Rotenone from 1-day post-eclosion and monitored daily for survival for up to 40-days. In order to determine the effect of rotenone exposure on locomotor ability negative geotaxis climbing assay was performed on the male flies fed on rotenone diet. Flies were tapped gently three times to the bottom of the vertical transparent tube (length, 25 cm; diameter, 3 cm) to initiate the negative geotaxis assay. After 10 s, the percentage of flies crossing the 5 cm mark was noted for each trial. A total of ten recordings were obtained per set and compared with untreated age matched control flies. All behavioural experiments were performed at room temperature under standard light conditions.

4.13 Resveratrol treatment

A dose-response curve was generated with varying conc. of resveratrol to monitor its effect on fly survival. Resveratrol was added to the fly food to achieve final concentration of 1 µM in the presence or absence of 200 µM rotenone. Flies were placed on rotenone containing food on the day of eclosion and transferred every other day to fresh vials and collected at 1 and 10-days post treatment.

4.14 Nicotinamide riboside (NR) treatment

A dose-response curve was generated with varying conc. of NR to monitor its effect on fly survival. NR was added to the fly food to achieve final concentration of 1 mM in the presence or absence of 100 and 200 µM rotenone. Flies were placed on rotenone containing food on the day of eclosion and transferred every other day to fresh vials and collected at 1, 10 and 20-days post-treatment.

4.15 Whole brain mount immunofluorescence labelling

The heads of adult flies were mechanically isolated under a dissection microscope and brains were dissected under stereo zoom microscope (Olympus, Japan). Immediately after dissection brain were fixed in 4% PFA for 30 min. Brain tissues were incubated overnight in anti-tyrosine hydroxylase (Merck Millipore) solution at 1:400 dilution in PBT (5% BSA and 0.3% Triton X-100 in PBS). Tissues were incubated with anti-rabbit Alexa 488 secondary antibody for Tyrosine hydroxylase at 1:500 dilution in PBT for 3 h to detect primary antibody. After immunostaining tissues were mounted in vectashield (Vector Laboratories). Whole brain images were taken in Apotome 2 microscope (Zeiss, Germany), 9–12 tiles were taken in motorized stage and in each tile's images of 5 slices of 0.32 µm were taken and image were reconstructed by ZEN software.

4.16 RNA Extraction:

Cells: For quantification of gene expression, cells were harvested and washed twice with 1X phosphate buffer saline (PBS) to completely remove medium. RNA was extracted using Purezol (Biorad, cat no: 732-6890) RNA isolation method. C-DNA was made using BioRad c-DNA synthesizing kit (Biorad, cat no: 1708841). 1.0 µg DNase (Thermo, cat no: EN0521) treated total RNA was used to make c-DNA.

Drosophila: For quantification of gene expression drosophila heads were dissected and homogenised. RNA was extracted using Purezol RNA isolation method. C-DNA was made using BioRad c-DNA synthesizing kit. 1.0 µg DNase-treated total RNA was used to make c-DNA.

4.17 Real time PCR analysis:

Real time analysis was performed for the specific target genes (Table 2) and was amplified from c-DNA with SYBR green PCR master mix (Biorad, cat no: 172-5125). 1ng of cDNA and 0.2µM primer in a total reaction of 10µl were used. The reaction was performed in 96 well plates at 95 ˚C for 3 min, followed by 40 cycles of 95 ˚C for 10 sec, 60 ˚C for 15 sec, 72 ˚C for 15 sec and melting curve 60 ˚C to 98 ˚C. Relative quantitative analysis was done using Cq value. GAPDH was used as endogenous control.

4.18 Western Blotting:

Cells: Cells were harvested and washed with ice-cold PBS and then lysed with M-PER mammalian protein extraction reagent (Thermo, cat no: 78503) containing protease inhibitor cocktail (Thermo, cat no: 78439). Cell debris removed by centrifugation at 12,000g for 15 minutes. The supernatant was quantified and normalised. Then western blot was done.

Drosophila: For quantification of gene expression drosophila heads were dissected and homogenised in lysis buffer. Protease inhibitor cocktail was added before lysis. Cell debris removed by centrifugation at 12,000g for 15 minutes. The supernatant was quantified and normalised followed by western blot analysis.

The specific antibodies, used in this study are listed in Table 3.

4.19 Statistical Analysis

Kaplan–Meyer survival analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). The results are presented as mean ± SEM (standard error of mean) of at least three independent experiments performed in triplicate. Statistical significance was measured by one-way analysis of variance (ANOVA). Asterisks indicate levels of significance (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

5. Results

Obejctive 1: *To understand the biochemical mechanisms of the role of SARM1 in mitochondrial electron transport chain (ETC) activity.*

5.1. The mitochondrial complex I inhibitor rotenone induce dose and time-dependent cell death in SH-SY5Y cells

Our first objective was to establish an in vitro cellular model system that would enable us to have an insight into the mechanism underlying Sarm1 mediated neuronal cell death. Since previous studies have indicated that Sarm1 localizes to the mitochondria upon stress and induce axonal retraction we chose the mitochondrial complex I inhibitor rotenone as our treatment of choice. The neuroblastoma cell line SH-SY5Y which has several characteristics of dopaminergic neurons, were treated with 2.5 and 5 µM of Rotenone for 24 h and 48 h. Cells showed signs of axonal retraction at 24 h post treatment without any damage in the cell body (Fig. 1A). Cell death following rotenone treatment was more prominent at 48 h post treatment (Fig. 1B). Hence, we envisioned that rotenone treatment to SH-SY5Y cells at 24 h post treatment could serve as an excellent model of studying axonal degeneration, a hallmark of several neurodegenerative diseases like Parkinson's disease.

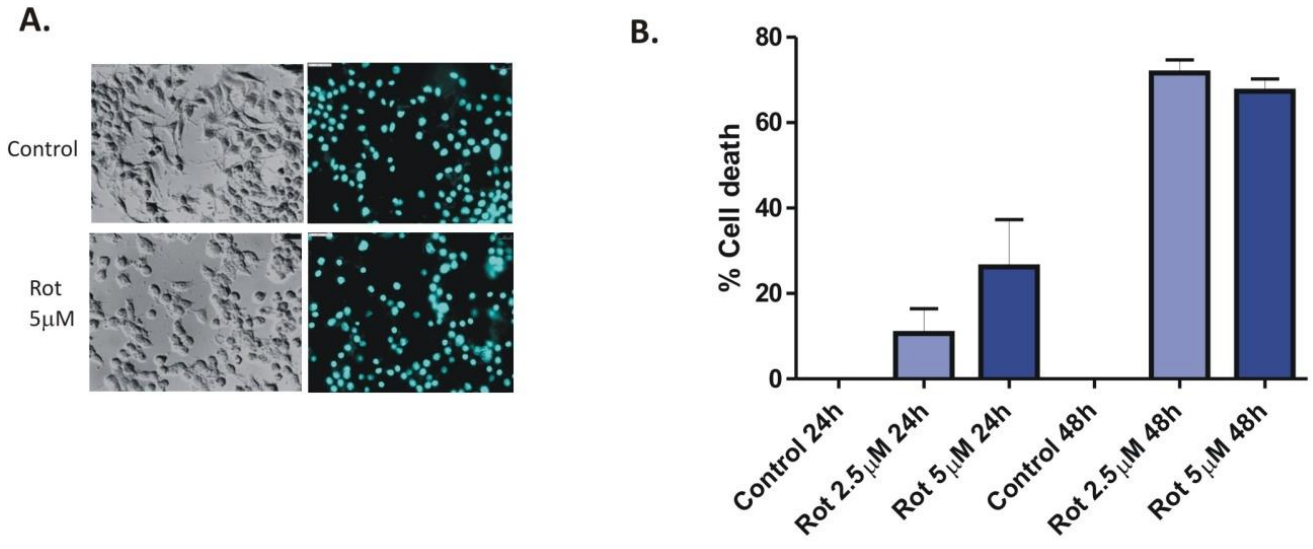


Fig. 1. *A Immunofluorescence analysis of SH-SY5Y cells treated with 5 µM of rotenone for 24 h and compared to untreated control cells. DAPI staining of both the treated and untreated samples are shown. B. MTT assay of SH-SY5Y cells treated with rotenone (2.5 and 5 µM) for 24 and 48 h. Results are representative of at least five independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.2. Rotenone treatment results in *Sarm1* induction in a time dependent manner

To understand whether rotenone treatment of SH-SY5Y cells resulted in Sarm1 induction, *Sarm1* gene expression was analyzed at 24 h post treatment. *Sarm1* gene expression significantly increased in both the whole cell extracts as well as in the nuclear and mitochondrial fraction following rotenone treatment (Fig. 2A and B). Since Sarm1 was previously shown to localize to the mitochondria upon stress we assessed Sarm1 localization in both the nuclear and mitochondrial fraction. We observed Sarm1 localization both at the nuclear and mitochondria with more prominent localization at the mitochondria (Fig. 2C) indicating that Sarm1 may mediate mitochondrial dysfunction following rotenone treatment.

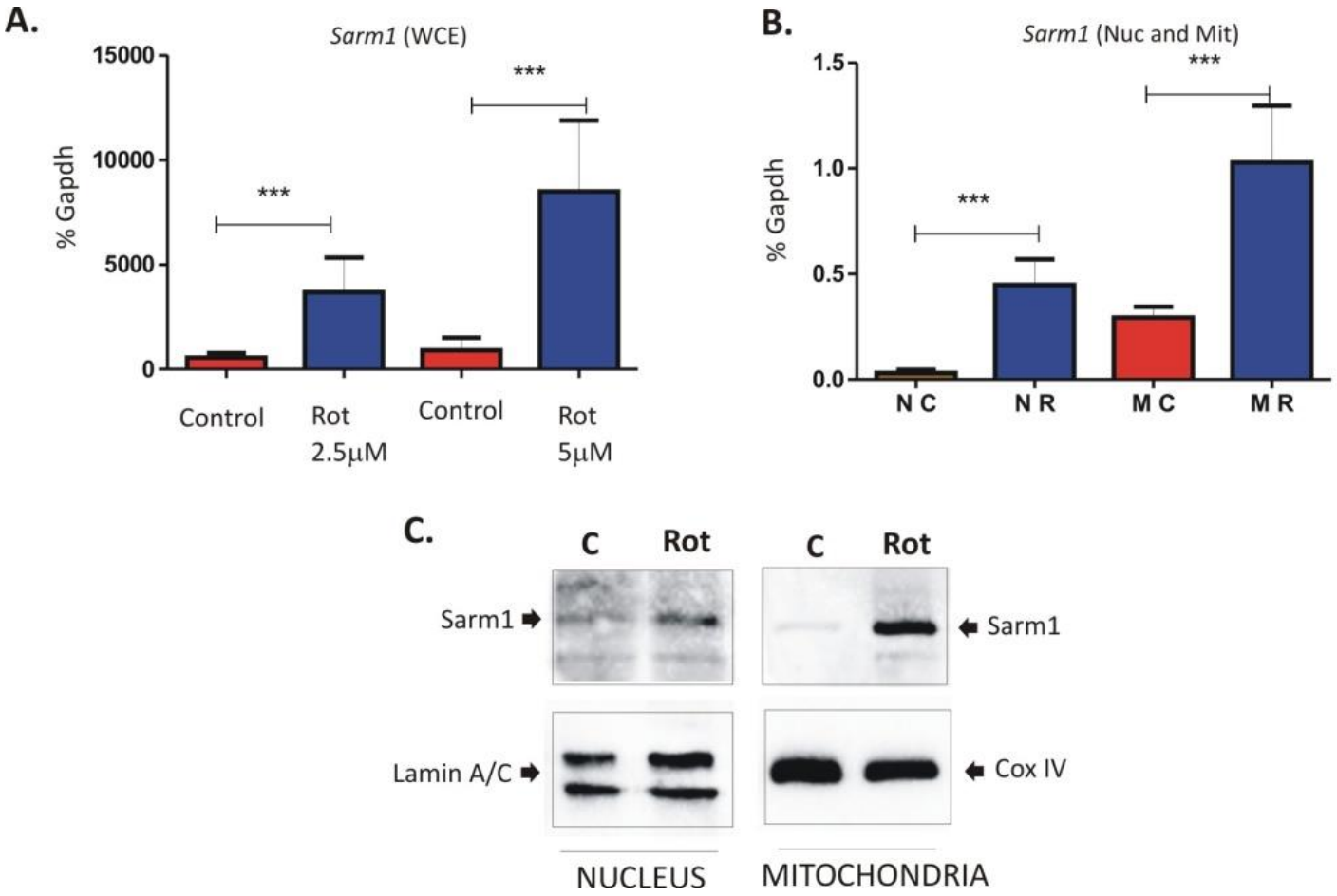


Fig. 2. (*A-B) Real time PCR analysis of SH-SY5Y cells treated with 2.5 and 5µM of rotenone for 24 h. A Expression of Sarm1 from whole cell extracts and B. nuclear and mitochondrial fractions were analysed. (C) Analysis of SARM1 protein levels in the nuclear and mitochondrial fractions of rotenone (5µM) treated SH-SY5Y cells. Lamin and COX IV were used as loading control for the nuclear and mitochondrial fraction respectively. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.3. Sarm1 is required for rotenone induced cell death in SH-SY5Y cells

To test whether Sarm1 was required for rotenone induced cell death, cells were transfected with siSarm. Our results indicate that prior treatment with siRNA for *Sarm1* followed by rotenone treatment significantly reversed cell death (Fig. 3A) and SARM1 protein levels were also reduced in these cells (Fig. 3B) indicating that SARM1 is the key mediator of the rotenone induced neurotoxicity in these cells.

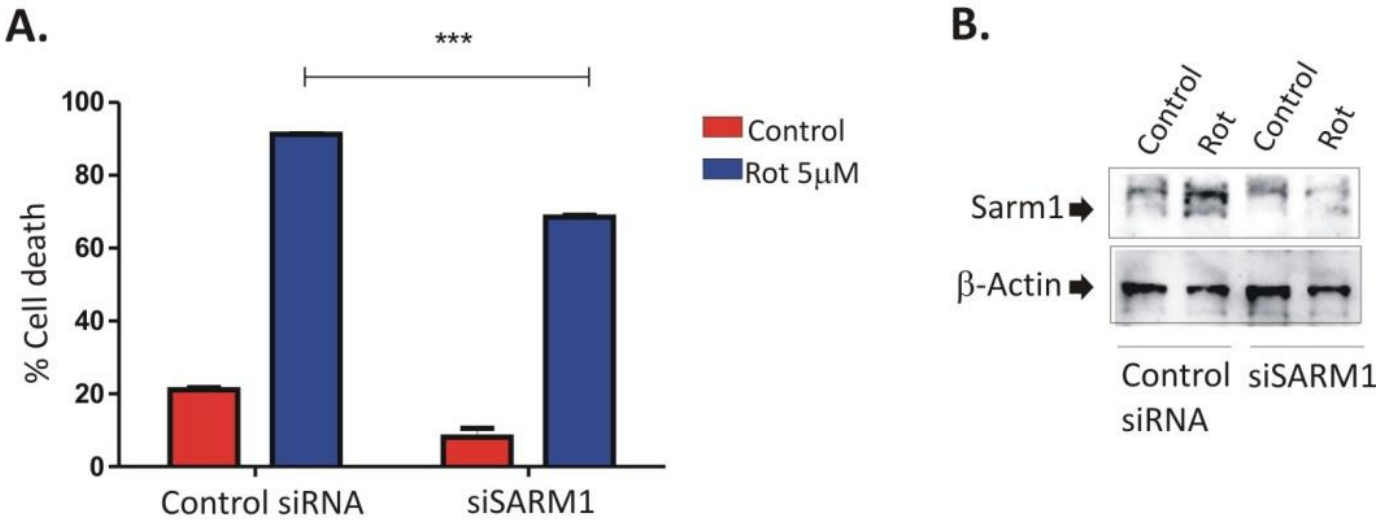


Fig. 3. (*A) SH-SY5Y cells were treated with 25 nM of Sarm1 siRNA or control (nontargeting) siRNA for 24 h followed by 5 μM of rotenone treatment for 24 h. A MTT assay of cells treated with rotenone in the presence or absence of siRNAs was performed. (B) Analysis of SARM1 protein levels in the whole cell extracts of cells transfected with Sarm1 or control siRNA in the presence or absence of rotenone (5 μM). β-actin was used as a loading control. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.4. Rotenone treatment results in de-regulation of ETC complex genes in SH-SY5Y cells

To understand the effect of rotenone treatment on the expression of the ETC complex genes and the role of SARM1, the expression of several genes of mitochondrial complex I, Complex III, Complex IV and Complex V were analysed in the whole cell extracts after 24 h treatment with rotenone. Rotenone treatment resulted in the deregulation of several ETC complex genes with significant increase in the expression of *Ndufs2*, *Cox5B* and *ATP12A* which are transcribed in the nucleus (Fig. 4).

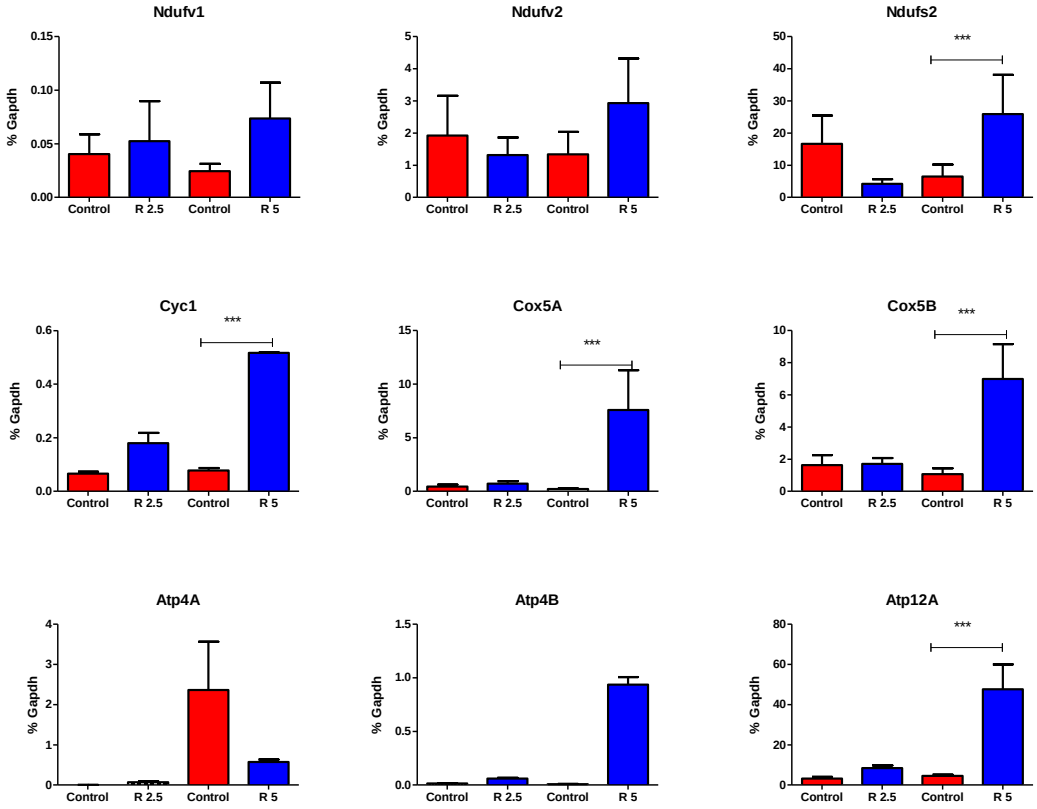


Fig. 4*. Real-time PCR analysis of SH-SY5Y cells treated with 2.5 and 5 µM of rotenone for 24 h. Expression of ETC complex genes from whole cell extracts were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control samples.*

Interestingly, *Ndufs2* and *Cox5B* showed specific upregulation in the nucleus and not the mitochondria (Fig. 5).

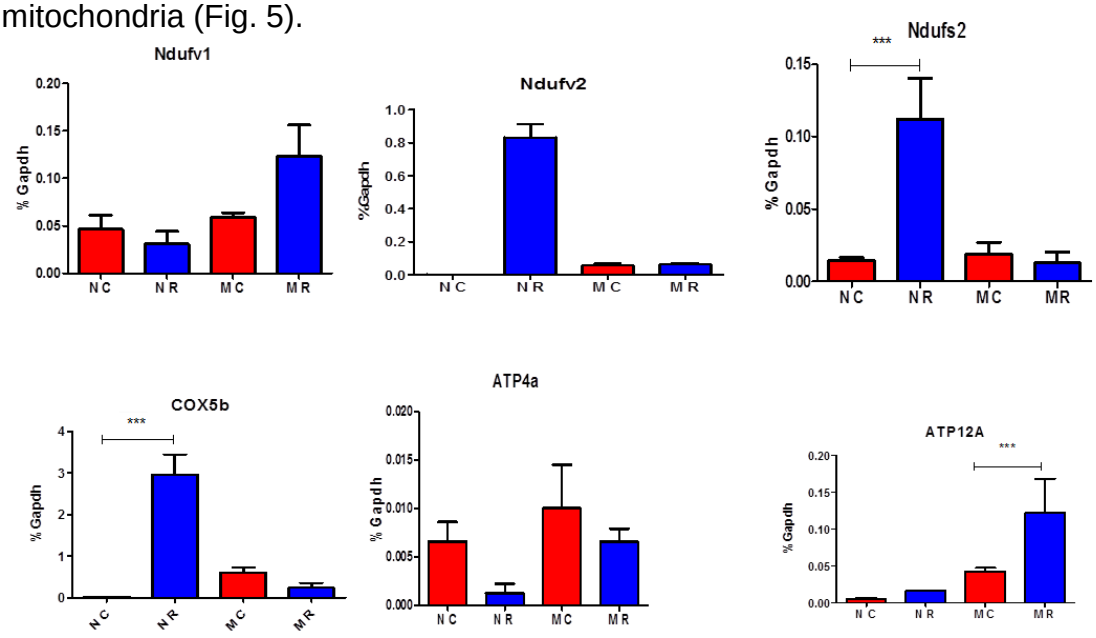


***Fig. 5**. Real-time PCR analysis of SH-SY5Y cells treated with 5 µM of rotenone for 24 h. Expression of ETC complex genes from nuclear and mitochondrial fractions were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.5. Rotenone induced deregulation of the respiratory complex genes is partially reversed in the presence of nicotinamide riboside (NR), a precursor of NAD^+ and the calcium channel blocker Nimodipine

Recent studies have shown that the TIR domain of Sarm1 has intrinsic NADase activity that may cause gross energy deficit within the cells ultimately leading to neurodegeneration. To determine whether SARM1 mediated axon degeneration could be blocked by the external supply of NR, SH-SY5Y cells were treated with NR (0.5 mM) along with rotenone (5 µM) to check the effect of NR in SARM1-mediated cell death following rotenone treatment. A significant reversal of rotenone mediated cell death was observed (Fig. 6A) along with the reduction in *Sarm1* gene expression (Fig.6B).

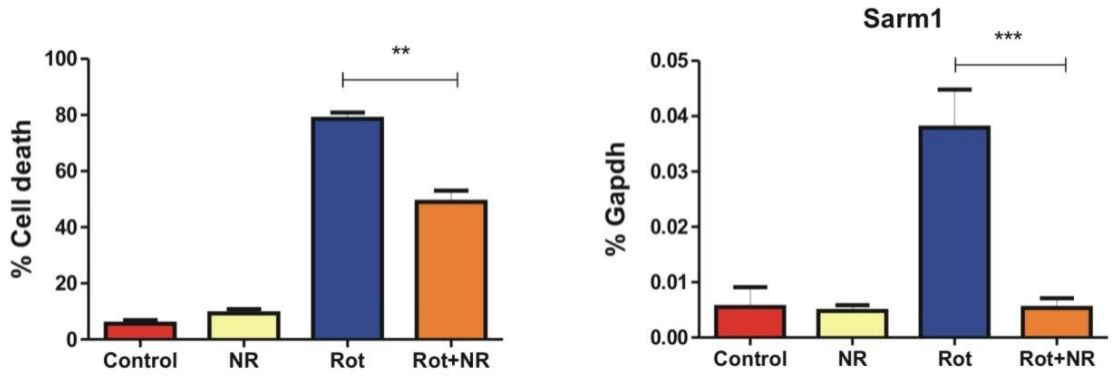


***Fig. 6.** MTT assay of cells treated with (A) rotenone (5 µM) and NR (0.5 mM) for 24 h. (B) Real-time PCR analysis of SHSY5Y cells treated with rotenone (5 µM) and NR (0.5 mM) for 24 h. Expression of Sarm1 from whole cell extracts was analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Rotenone mediated deregulation of ETC complex gene expression was also reversed in case of NR treatment (Fig. 7).

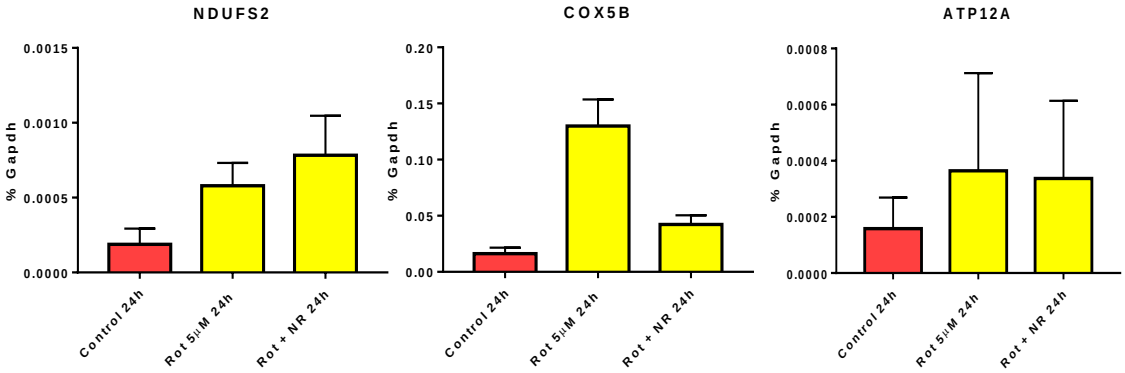


Fig. 7. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and NR (0.5 mM) for 24 h. Expression of inflammatory and ETC complex genes from whole cell extracts were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

It has been shown that SARM1 functions downstream of the axonal calcium increases after axotomy, as in *C.elegans* Tir-1 (homolog of human SARM1) acts downstream of the Ca^{2+}-CaM kinase signalling pathway. In order to understand whether the calcium acts upstream of SARM1 induction and plays a major role in SARM1 mediated cell death, SH-SY5Y cells were treated with 10 µM of nimodipine (calcium channel blocker) along with 5 µM of rotenone and a significant reversal of the *Sarm1* expression was observed following nimodipine treatment (Fig. 8).

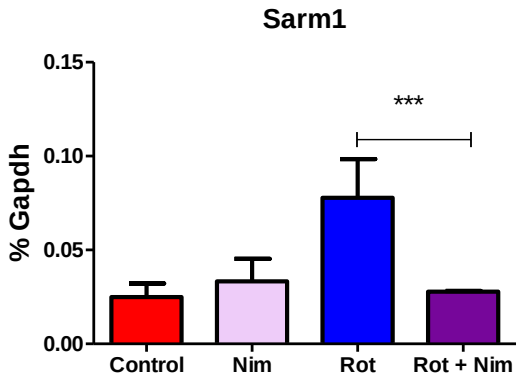


Fig.8. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and nimodipine (10 µM) for 24 h. Expression of SARM1 from whole cell extracts was analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Rotenone mediated deregulation of ETC complex gene expression were also reversed following nimodipine treatment indicating Sarm1 expression may have correlation with these ETC complex genes (Fig.9).

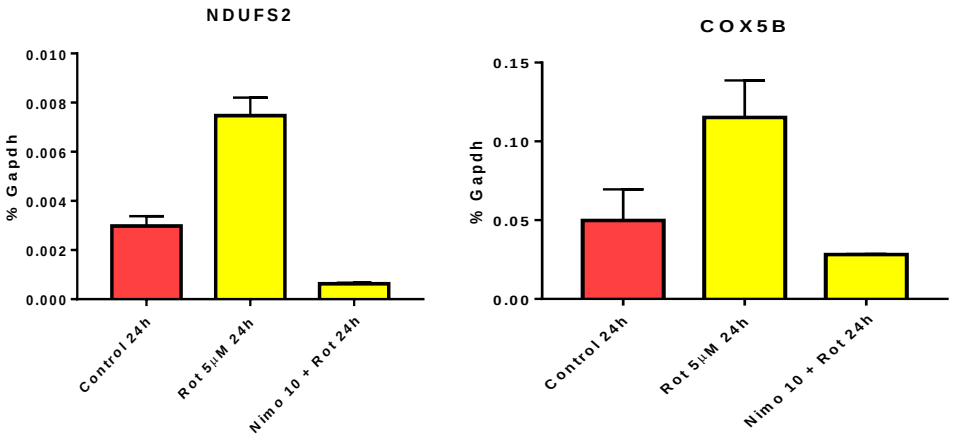


Fig.9. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and nimodipine (10 µM) for 24 h. Expression of ETC complex genes from whole cell extracts was analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Interestingly, the levels of Sarm1 remained high in the nucleus in the presence of nimodipine (Fig. 10).

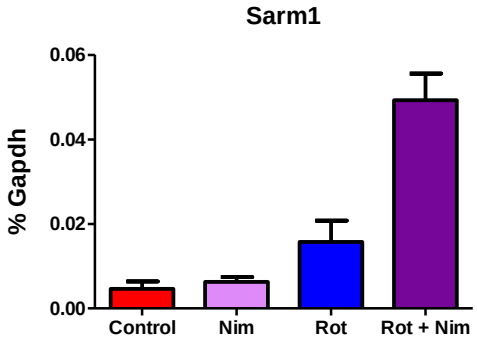


Fig.10. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and nimodipine (10 µM) for 24 h. Expression of SARM1 from whole nuclear fraction was analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

To further confirm the role of calcium in the neurodegeneration, the calcium ion chelator EGTA (2.5 mM) was added in the presence or absence of rotenone.

As observed previously a significant reversal of the *Sarm1* expression (Fig. 11) was found along with a delayed rotenone induced cell death.

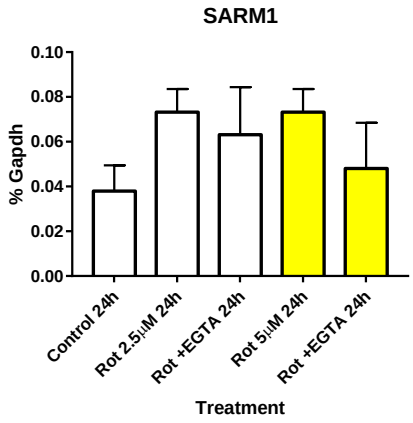


Fig.11. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and EGTA (2.5 mM) for 24 h. Expression of SARM1 from whole cell extracts was analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.6. Nicotinamide riboside (NR) reduces *Sirtuin* gene expression in rotenone treated cells

Since the NAD^+ precursor NR reversed the deregulation of ETC complex genes induced by rotenone, we speculated that NAD^+ levels may have a role on the mitochondrial sirtuins that uses NAD^+ as a co-factor. Further, a few reports suggest that sirtuins may play an important role in the deacetylation of the ETC complex genes and thus regulated their activity. We observed a significant upregulation of the expression of *Sirtuin* genes that was reversed in the presence of NR (Fig.12).

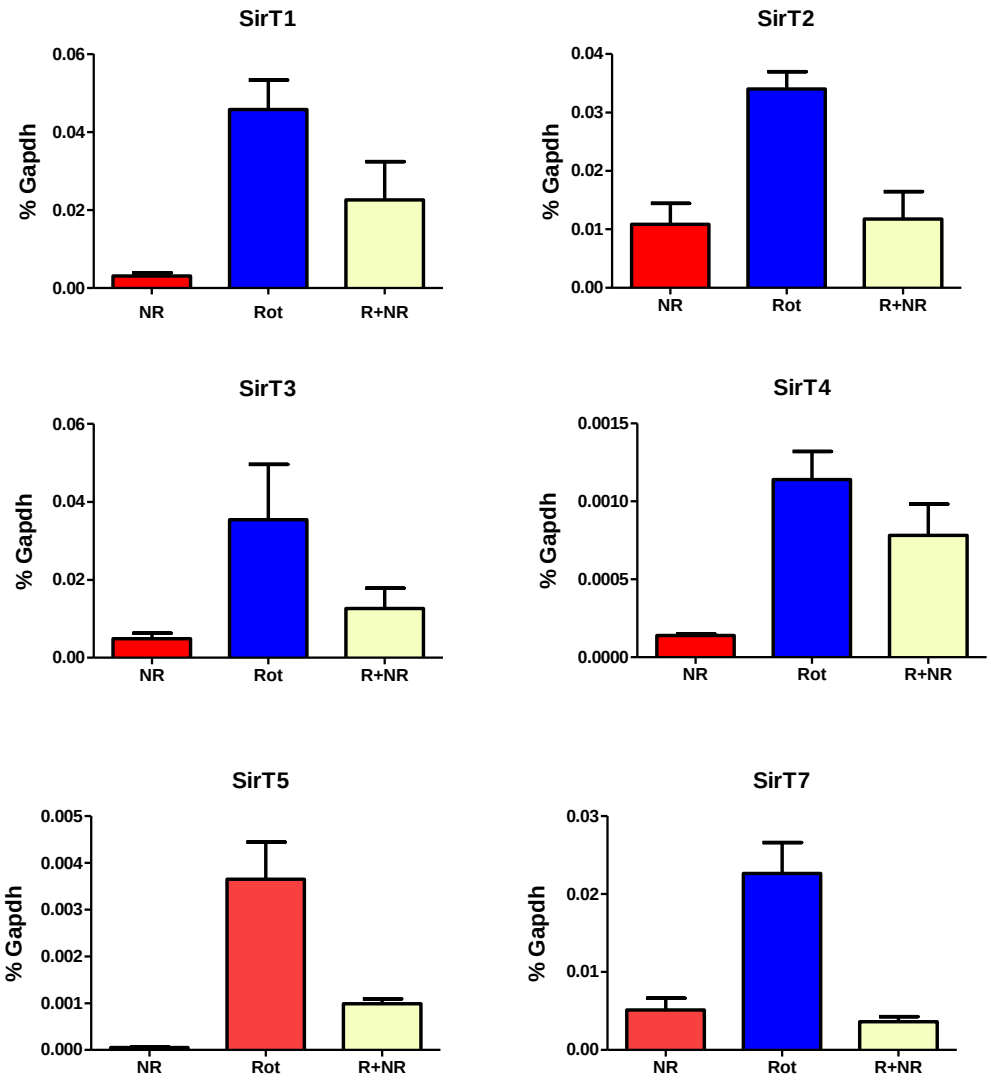


Fig.12. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and NR (0.5 mM) for 24 h. Expression of Sirtuin genes from whole cell extracts were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Conclusion

Our results indicate that rotenone treatment induces Sarm1 and its localization to the mitochondria. Sarm1 is required for rotenone induced cell death and correlates with the deregulation of the ETC genes especially those that are transcribed in the nucleus. Pre-incubation with the NAD^+ precursor nicotinamide riboside reverses rotenone induced cell death and also reduces *Sarm1* gene expression and the ETC genes. Further, NR treatment reduces the heightened expression of Sirtuins that may play an important role in the regulation of mitochondrial homeostasis, the mechanism of which needs to be explored further.

Obejctive 2: *To determine the site of ROS generation by analyzing the formation of respiratory complexes*

5.7. Rotenone treatment in SH-SY5Y cells is accompanied by increased ROS generation

Rotenone treatment results in early induction of mitochondrial ROS. A time-dependent study was carried out and it was found that rotenone induces ROS generation as early as 3 and 4 h post treatment (Fig. 13A) prior to axonal retraction and subsequent cell death. Cells were also stained with Mitosox red and it was evident that there was an early increase in mitochondrial ROS (Fig.13B). However, treatment of cells with the antioxidant N-Acetyl cysteine (NAC) reduced the levels of ROS (Fig. 13C) but did not reverse rotenone induced toxicity (Fig. 13D) which indicates that ROS may not be the sole player in rotenone mediated neuronal cell death.

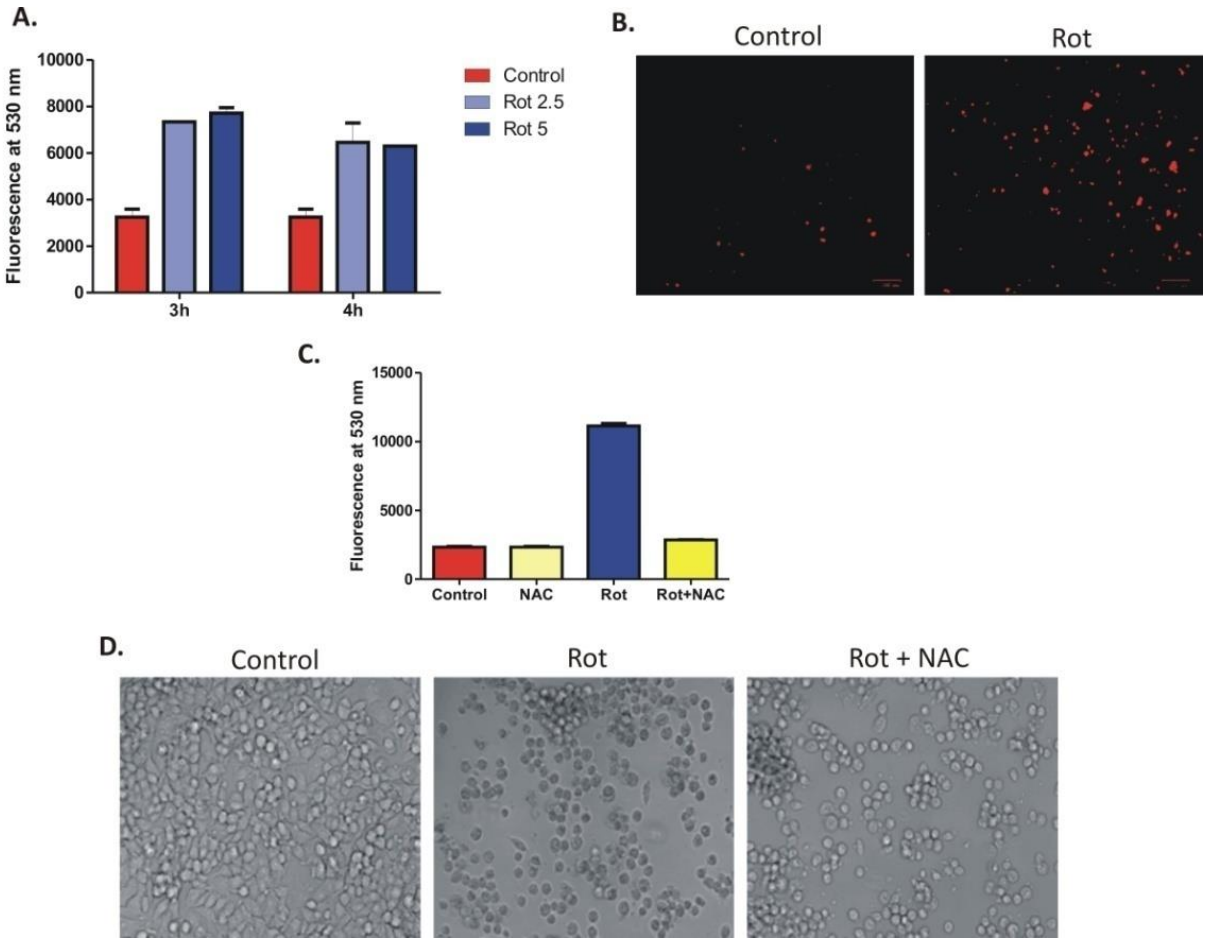


***Fig. 13**. (A) ROS generation assay of SH-SY5Y cells treated with 2.5 and 5 µM of rotenone for 3 and 4 h. (B) Mitosox staining analysis of SH-SY5Y cells treated with 5 µM of rotenone. (C) ROS generation assay of SH-SY5Y cells treated with 5 µM of rotenone and 5 µM of NAC. (D) Axonal retraction of SH-SY5Y cells treated with 5 µM of rotenone and 5 µM of NAC for 24 h. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.8. SARM1 is not induced in the presence of other mitochondrial inhibitors that generate ROS

To further understand the specific role of SARM1 in rotenone mediated neurotoxicity and the role of mitochondrial dysfunction, cells were exposed to Antimycin, Oligomycin and FCCP (Complex III and V inhibitor and uncoupler of mitochondrial oxidative phosphorylation respectively). Treatment of SH-SY5Y cells with 5.0 µM of antimycin, 10.0 µM of oligomycin and 2.0 µM of FCCP for 24 h resulted in cell death (Fig. 14A-C) with no signs of axonal retraction as observed in rotenone mediated toxicity.

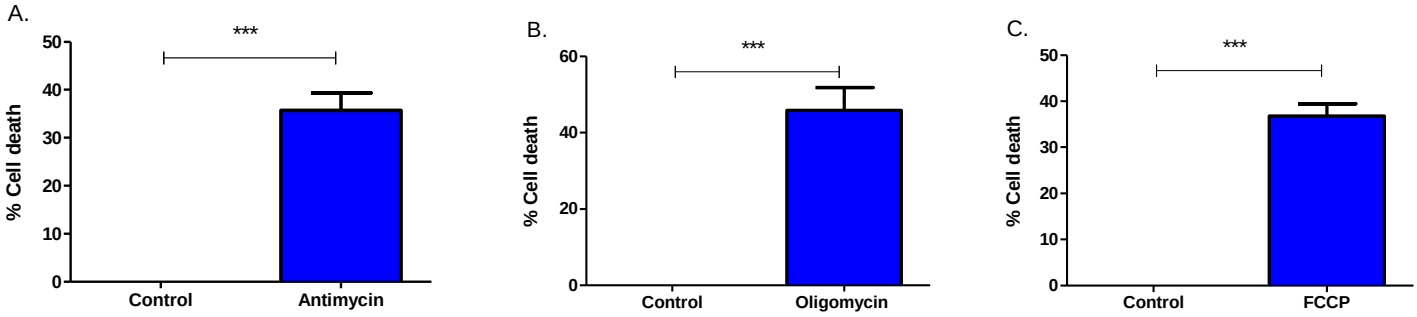


***Fig. 14**. (A-C) *MTT assay of cells treated with (A) antimycin (5 µM) (B) oligomycin (10µM) and (C) FCCP (2 µM) for 24 h. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

A time-dependent study was carried out and it was found that these inhibitors induced ROS generation as early as 6 post treatment (Fig. 15A and B).

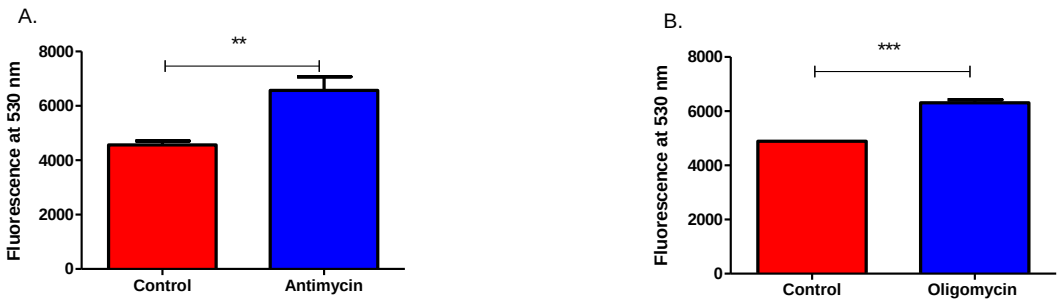


***Fig. 15**. ROS generation assay of cells treated with (A) antimycin (5 µM) and (B) oligomycin (10µM) for 6 h. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Interestingly, these inhibitors did not induce the expression of *Sarm1* and the ETC complex genes were differentially regulated as compared to rotenone treatment (Fig. 16).

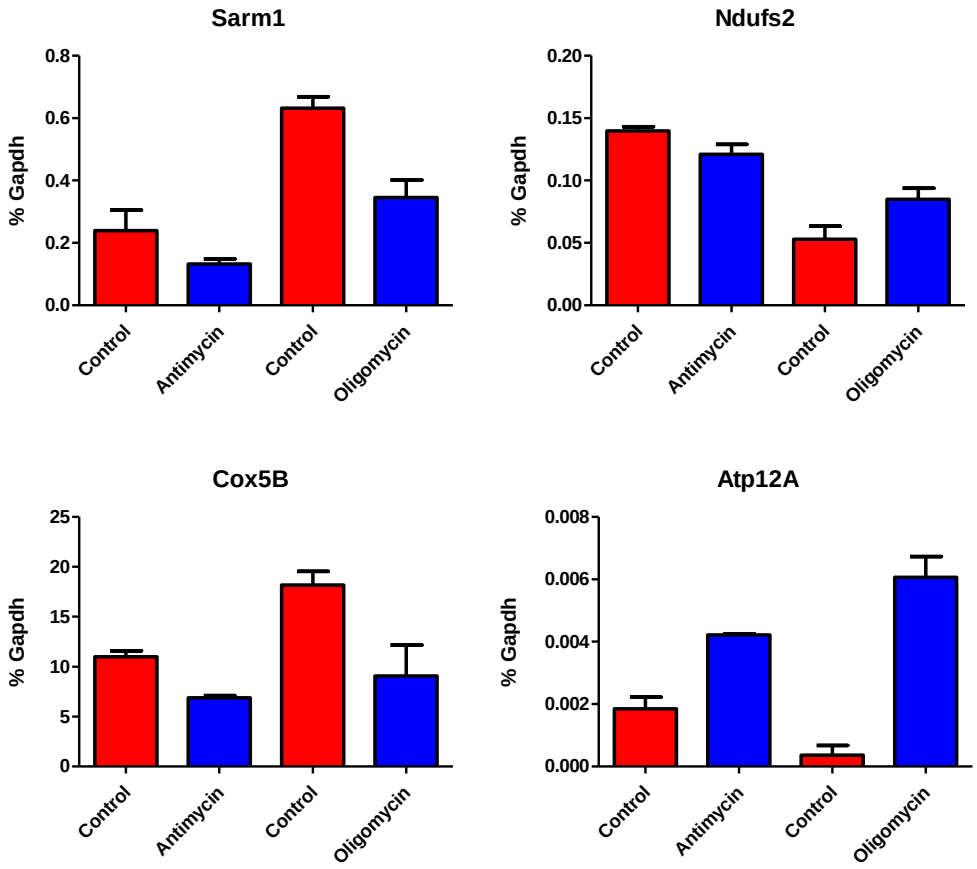


***Fig.16**. Real-time PCR analysis of SH-SY5Y cells treated with antimycin (5 µM) and oligomycin (10 µM) for 24 h. Expression of SARM1 and ETC complex genes from whole cell extracts were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.9. Rotenone inhibits autophagic flux that may result in Sarm1 induction in SH-SY5Y cells

Since treatment of cell with the antioxidant NAC did not reverse cell death nor Sarm1 induction, we hypothesized there could be other forms of stress within the cells that may lead to the upregulation of Sarm1 and subsequent cell death. Representative western blot indicates that rotenone treatment does not increase levels of the apoptotic substrate caspase at 3 h (Fig, 17A) but at 24 h (Fig. 17B), indicates occurrence of apoptosis at later phase. Since axonal retraction occurred prior to Sarm1 induction we explored pathways that may lead to Sarm1 induction and cellular apoptosis.

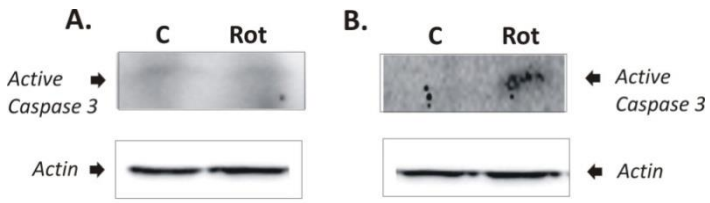


Fig.17. *Analysis of Caspase 3 protein levels in the whole cell extracts of cells treated with rotenone (5 μM) at 3 h (A) and 24 h (B). β-actin was used as a loading control. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

We analysed several autophagy related genes as 3 h, 6 h and 24 h post treatment and found an initial increase in *Atg* genes (Fig. 18 A-C). However, the expression of genes involved in autophagosome-lysosome fusion was downregulated at all the time points.

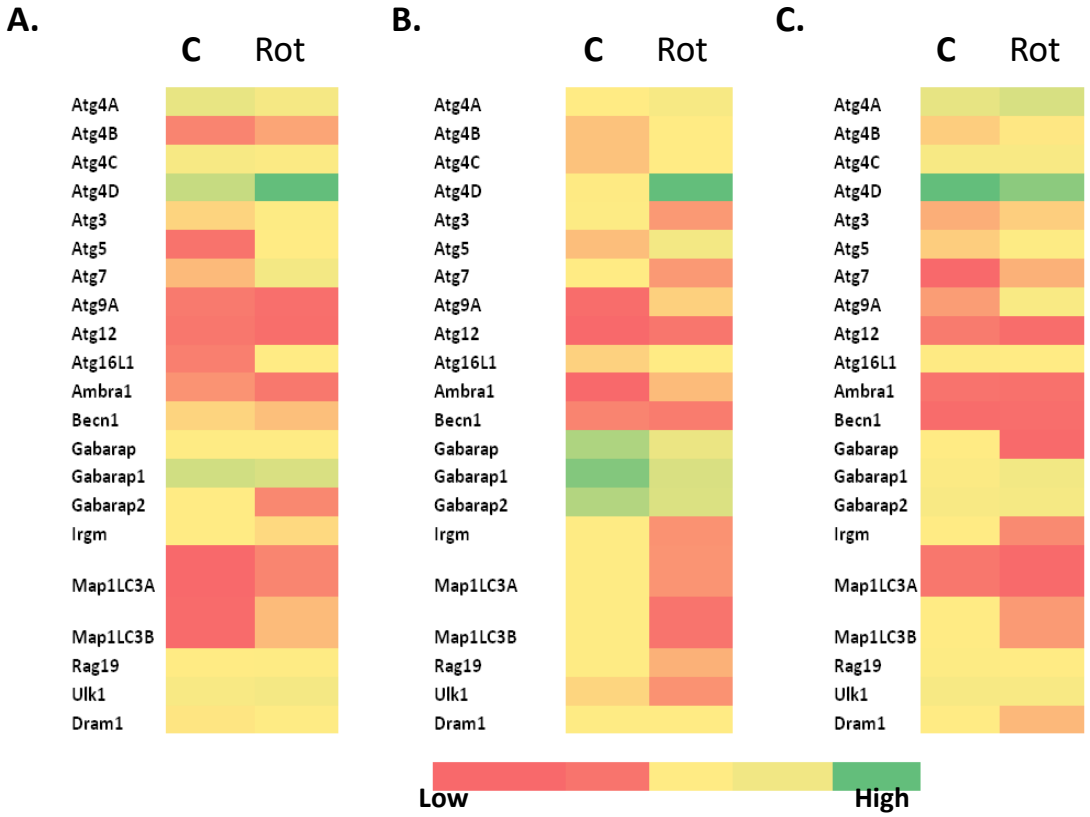


Fig.18. *Heat Map analysis of cells as treated with rotenone (5 μM). Autophagy related genes were analysed following 3 h (A), 6 h (B) and 24 h (C) post treatment.*

A similar deregulation of the autophagy genes was observed in the mitochondrial fraction of rotenone treated cells (Fig. 19). Interestingly, the genes were restored to normal levels following NR treatment.

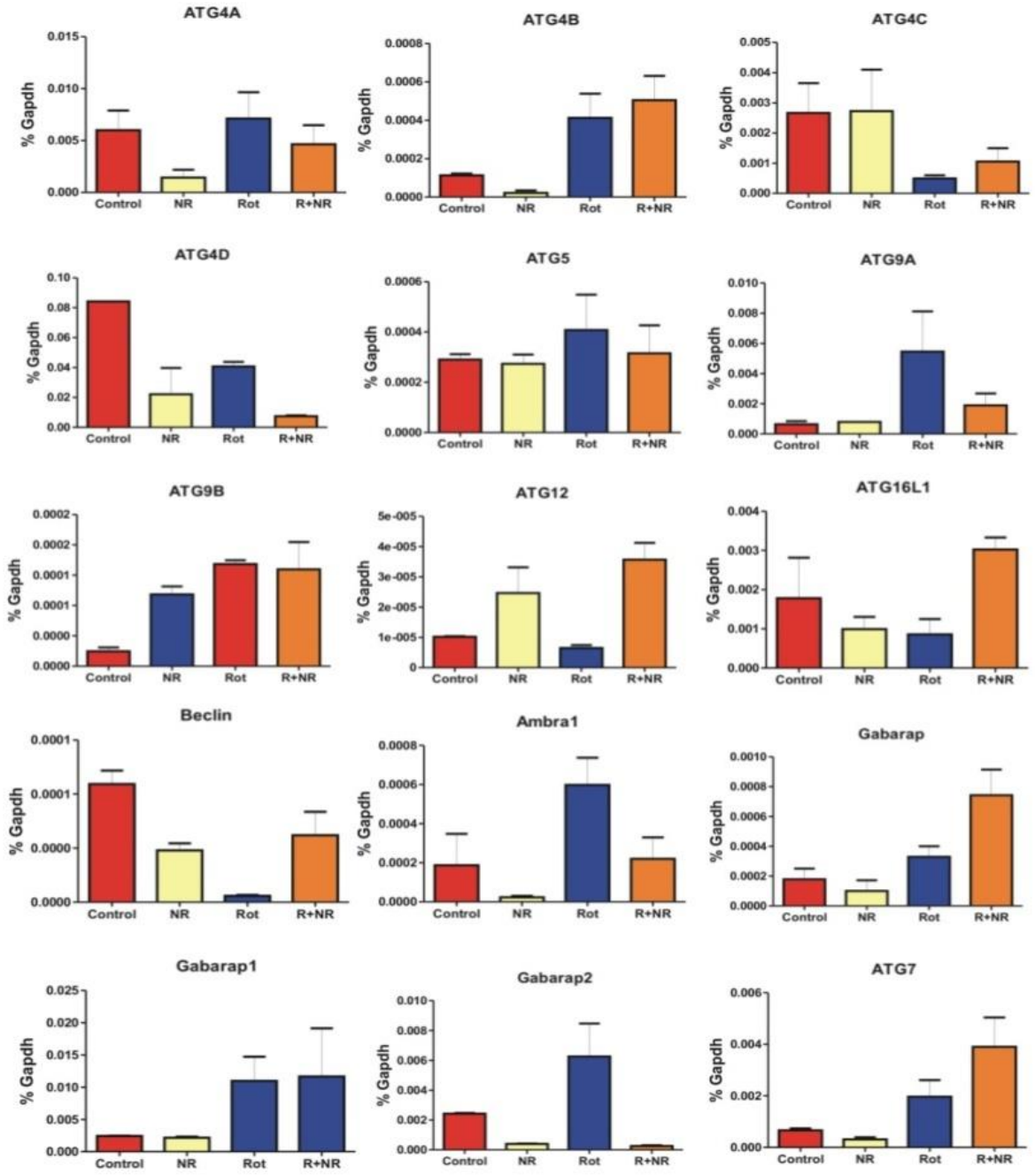


Fig 19. *Real-time PCR analysis of SH-SY5Y cells treated with rotenone (5 µM) and NR (0.5 mM) for 24 h. Expression of autophagy related genes from mitochondrial fraction were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Analysis of the key autophagy proteins showed accumulation of both p62 and the increased levels of LC3B indicating that rotenone blocked the autophagic flux in these cells (Fig. 20).

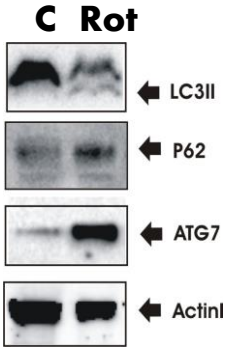
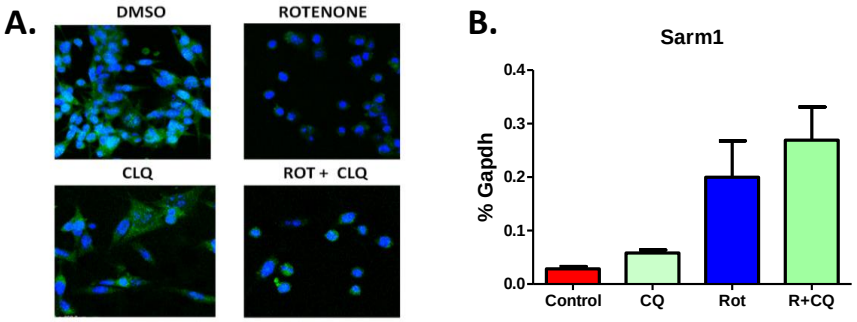


Fig.20. *Analysis of p62, ATG7 and LC3B protein levels in the whole cell extracts of cells treated with rotenone (5 µM) at 24 h. β-actin was used as a loading control.*

Treatment with the autophagy inhibitors had opposing effects. Chloroquine increased rotenone mediated cell death (Fig. 21) along with higher Sarm1 expression.



However, Bafilomycin reversed rotenone mediated cellular toxicity that was accompanied by reduced *Sarm1* gene expression (Fig. 22).

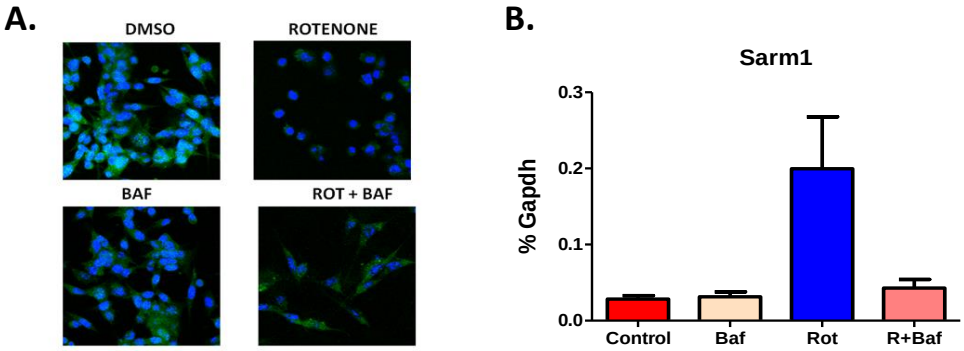


Fig.22. *(A) Immunofluorescence analysis of SH-SY5Y cells treated with 5 µM of rotenone and Chloroquine (100 µM) for 24 h and compared to untreated control cells. DAPI and MAP2 staining of both the treated and untreated samples are shown. (B) Real-time PCR analysis of SHSY5Y cells treated with rotenone (5 µM) and bafilomycin (100 nM) for 24 h. Expression of SARM1 from whole cell extracts were analysed. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Conclusion

Our results indicated that rotenone treatment resulted in increased ROS generation. However, contrary to our hypothesis we found that pre-treatment with the antioxidant NAC reduced rotenone induced ROS levels but did not reverse *Sarm1* expression or accompanying cell death. Hence we explored other pathways that may increase cellular stress level and lead to Sarm1 mediated apoptosis. Removal of damaged mitochondria by the process of autophagy is an important cellular mechanism for maintaining mitochondrial homeostasis and preventing ROS accumulation in the cells. Our results showed that rotenone treatment resulted in blockage of autophagic flux and further inhibition of autophagy by the autophagy inhibitor chloroquine resulted in significant cell death that was accompanied by much higher *Sarm1* expression. Interesting, treatment with another autophagy inhibitor Bafilomycin had opposing effect and it can be speculated that Sarm1 might interact with the H+-ATPase and block the function of Bafilomycin. To further explore the pathways involved RNA sequencing analysis are underway to determine the effect of Bafilomycin in rotenone treated cells.

Obejctive 3: *To investigate the effect of excess ROS generation on mitochondrial homeostasis and susceptibility to environmental stress in aging*

5.10. Rotenone exposure causes reduced lifespan and progressive locomotor deficits in w^{1118} strain of Drosophila in an age-dependent manner

Studies in drosophila model have shown that chronic rotenone exposure of flies could result in dopaminergic neuronal loss accompanied by locomotor deficits and increased generation of ROS. However, these studies fail to explain the fact that replenishing dopamine relieve initial symptoms but cannot establish a long lasting cure to the motor deficits observed in PD which is probably associated with rapid neuronal loss at later stage of the disease. Further, these studies examined the effect of chronic rotenone exposure in younger flies (7-days following exposure) but not the age-dependent vulnerability to low level chronic exposure to such environmental toxins. Thus, an in-depth analysis of the mechanisms underlying the loss of dopaminergic neurons and how age acts as a trigger for the increased susceptibility to loss of these neurons is required to explain this phenomenon.

To study the effect of the mitochondrial complex I inhibitor rotenone on Drosophila survival, w^{1118} flies were exposed to increasing doses of rotenone 1-day post-eclosion and survival of the flies were followed up to 40-days as represented in Fig. 23A. As shown in Fig. 23B the flies were susceptible to increasing concentration of rotenone (50, 100 and 200 µM) that was accompanied by increased motor deficits as shown by their decreased climbing ability in a negative geotaxis assay at 1-day (Fig. 23C), 10-days (Fig. 23D) and 20-days (Fig. 23E) post-treatment. One-way ANOVA indicates that climbing ability in 200 µM rotenone exposed adult w^{1118} flies was significantly ($P < 0.001$) less than that of untreated flies after 1-day, 10-days and 20-days rotenone exposure.

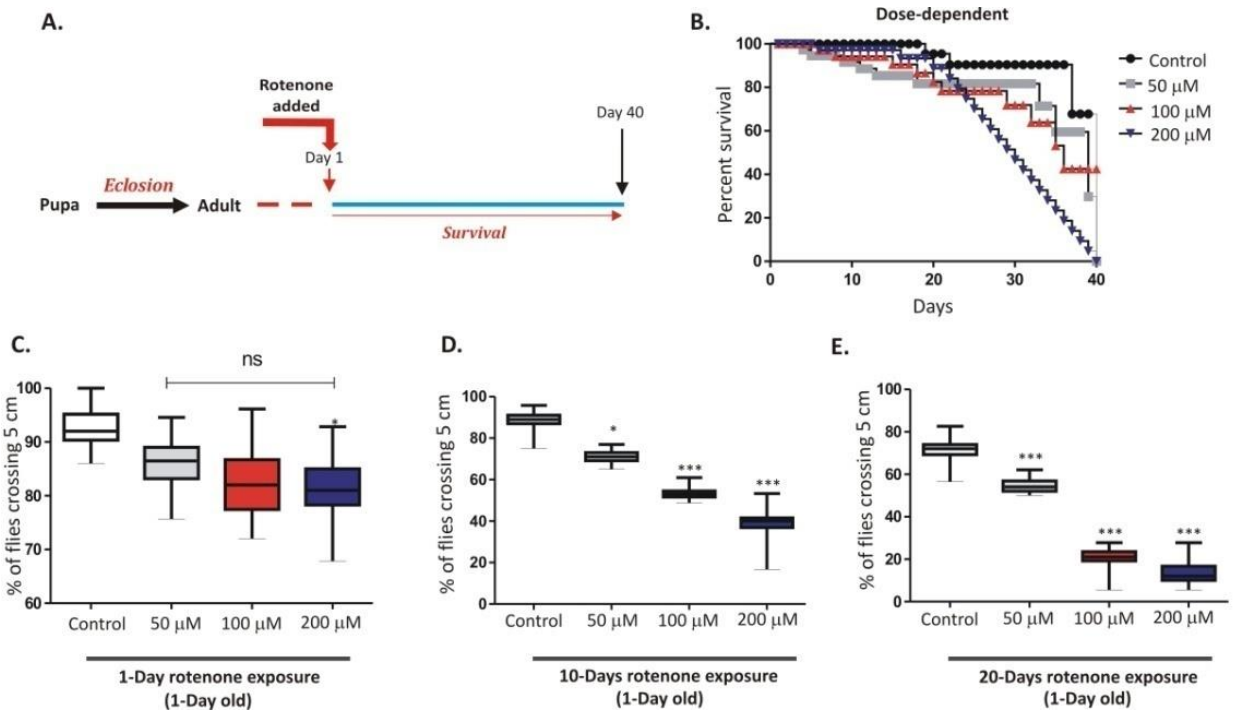


Fig.23. *(A) Schematic representation of rotenone treatment in the 1-day old flies for survival curve assay. (B) Survival curve of 1-day old flies exposed to varying concentration of rotenone (50, 100, and 200 µM). Fly viability was scored over a period of 40-days using a minimum of 100 flies per treatment. The statistical significance was calculated as log-rank using Mantel–Cox test. (C–E) Negative geotaxis assay of 1-day old flies exposed to rotenone at concentrations indicated above for 1-day (C), 10-days (D) and 20-days (E). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

To determine the age-related susceptibility to environmental toxins, flies were exposed to the sub-lethal concentration (100 µM) of rotenone at day-1, day-10 and day-20 following eclosion (Fig. 24A). Interestingly, age played a significant role in the increased susceptibility of these flies to rotenone exposure. Flies exposed to rotenone at 20-days following eclosion (older flies), the death rate was significantly increased ($P < 0.001$) compared to the younger flies (Fig. 24B) which was accompanied by severe motor deficits (Fig. 24C).

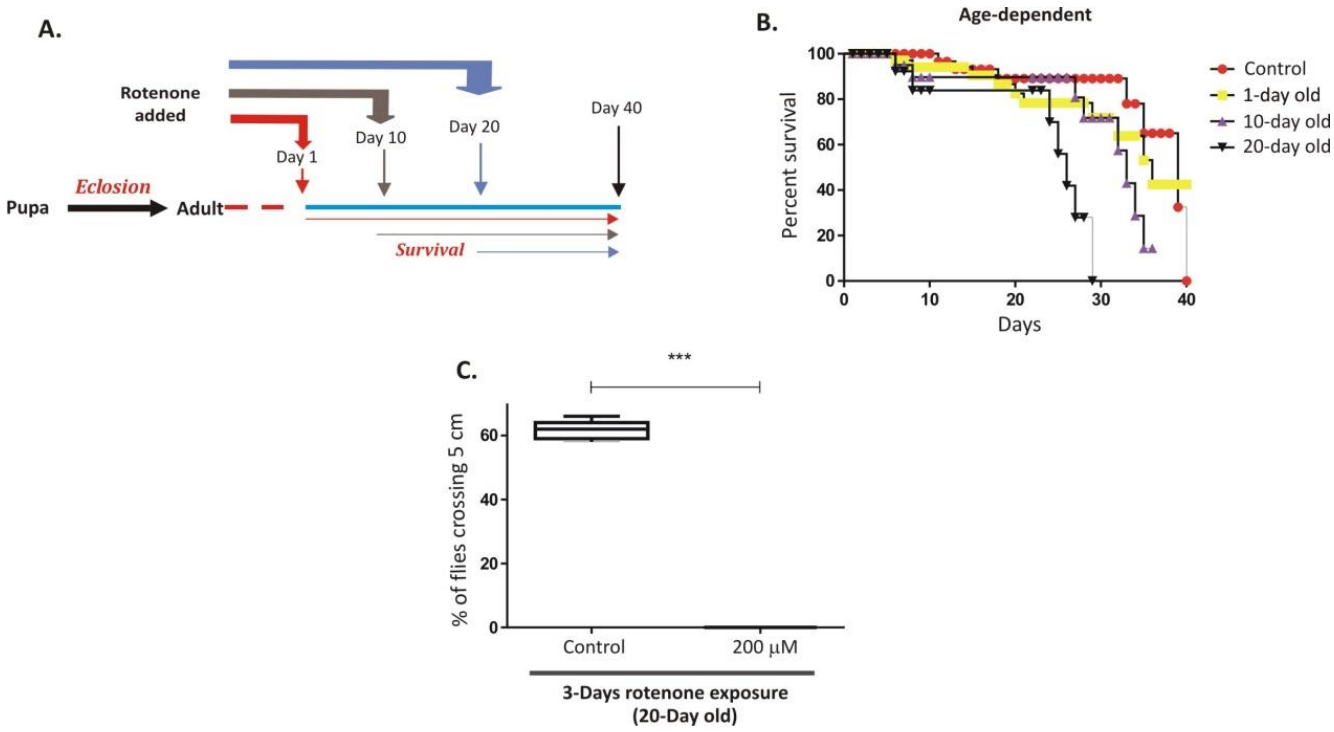


Fig.24. *(A) Schematic representation of rotenone treatment in the 1-day, 10-day, and 20-day old flies for survival curve assay. (B) Age-dependent survival of flies exposed to 100 µM of rotenone at 1-day, 10-day, and 20-day following eclosion. Fly viability was scored up to 40-days, post-eclosion using a minimum of 100 flies per treatment. (C) Negative geotaxis assay of 20-day old flies exposed to 200 µM rotenone for 3-days. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control flies*

5.11. Age-dependent susceptibility to rotenone is accompanied by increased dSarm expression

Clearly our data indicates that age is a risk factor for increased susceptibility to rotenone induced neurotoxicity. In an attempt to identify the molecular mechanism for this increased susceptibility we analysed the expression of one of the neurodegenerative molecules *dSarm* (Drosophila homolog of *Sarm1*) that has been implicated in injury induced axonal loss in flies in the young (1-day old) and aged (20-day old) flies as represented schematically in Fig. 25A. An early induction of *dSarm* was observed in the brain of young flies as early as 10-days post exposure, which peaked at 20-days (greater than 3-fold increase compared to 1-day old flies) followed by a steep decrease at 30-days post exposure (Fig. 25B). To further understand the age-dependent susceptibility to rotenone, aged flies (20-day old) were exposed to 200 µM rotenone and brains were collected at 3-days and 5-days post exposure. These flies were highly susceptible to rotenone exposure and did not survive beyond 10-days post exposure. As compared to the younger flies there was a significant increase in *dSarm* expression (Fig. 26C) as early as 3-days post exposure in these aged flies followed by a rapid decrease at 5-days which correlated with the increased age-related susceptibility to rotenone.

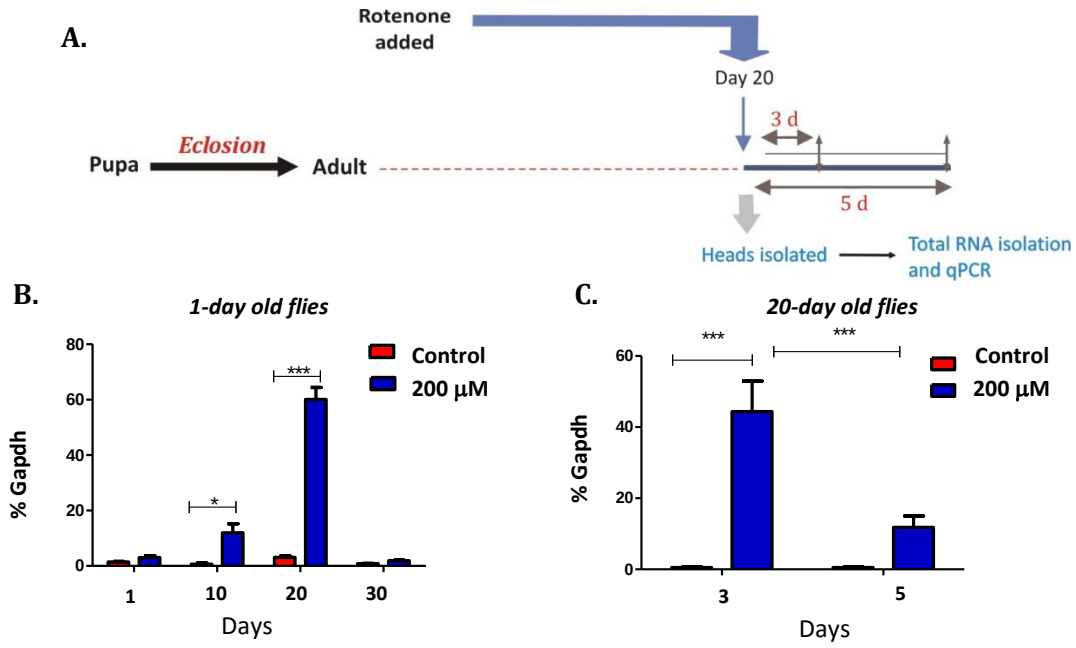


Fig.26. *(A) Schematic representation of experimental setup for rotenone treatment in the aged (20-days old) flies. B, C Expression of dSarm in young flies (1-day old) exposed to 200 µM of rotenone for 1-day, 10-days, 20-days, and 30-days (B) and aged flies (20-days old) to 200 µM of rotenone for 3-days and 5-days post-treatment (n = 5) (C). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

In agreement with our earlier data of age-related increased susceptibility to rotenone, we observed a rapid and significant loss of dopaminergic neurons from all the dopaminergic neuronal clusters indicated by tyrosine hydroxylase (TH) staining of whole brains in the 20-day old flies exposed to rotenone for 3-days as compared to age-matched control flies (Fig. 27A-B). We also observed a significant reduction of TH protein levels in the 20-day old flies as early as 3-days post exposure with 200 µM of rotenone as compared to their age-matched controls (Fig. 27C).

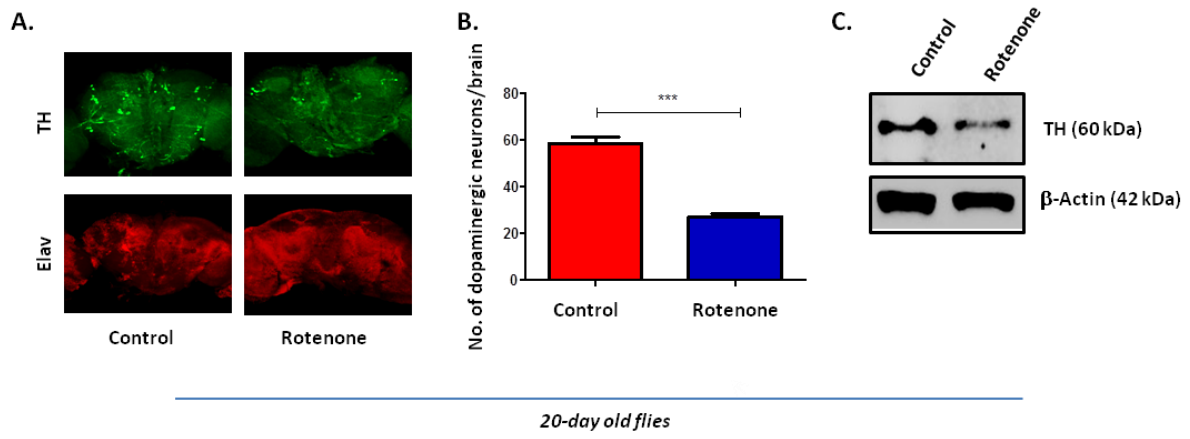


Fig.27. *(A) Multifocal confocal image of dopaminergic neurons following tyrosine hydroxylase (TH) immunostaining (green) and Elav immunostaining (red) in the brains of flies exposed to 200 µM rotenone at 20-days post-eclosion for 3-days (right-hand panel). Results were compared to age-matched rotenone untreated flies (left-hand panel). Scale bars = 200 µm. (B) Bar graph represents the number of dopaminergic neuron cell bodies in the control and rotenone (200 µM) treated fly brains (n = 3). (C) Representative western blot analysis of the dopaminergic neuronal marker tyrosine hydroxylase in 20-day old flies exposed to 200 µM rotenone and analysed at day-3 post-exposure. β-actin served as a loading control and data was compared with age-matched untreated control flies (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control flies*

5.12. An early induction of *dSarm* was found to be necessary and sufficient for locomotor deficits following exposure to rotenone

To understand whether the early induction of *dSarm* as observed in Fig. 26B was sufficient to initiate rotenone induced neurotoxicity, flies were exposed to 200 µM of rotenone for 10-days following which they were grown in media without rotenone (Fig. 28A). An early exposure to rotenone in the younger flies for 10 days and subsequent removal and growth of these flies in rotenone-free media continued to induce loss of survival (Fig. 28B) and locomotor deficits (Fig. 28C). A paired student t-test indicated significant (P <0.01) upregulation of *dSarm* expression following early rotenone exposure (Fig. 28D) in these flies. Taken together, these data suggest that early SARM1 induction may act like a switch to trigger the loss of dopaminergic neurons leading to rotenone induced motor deficits in flies.

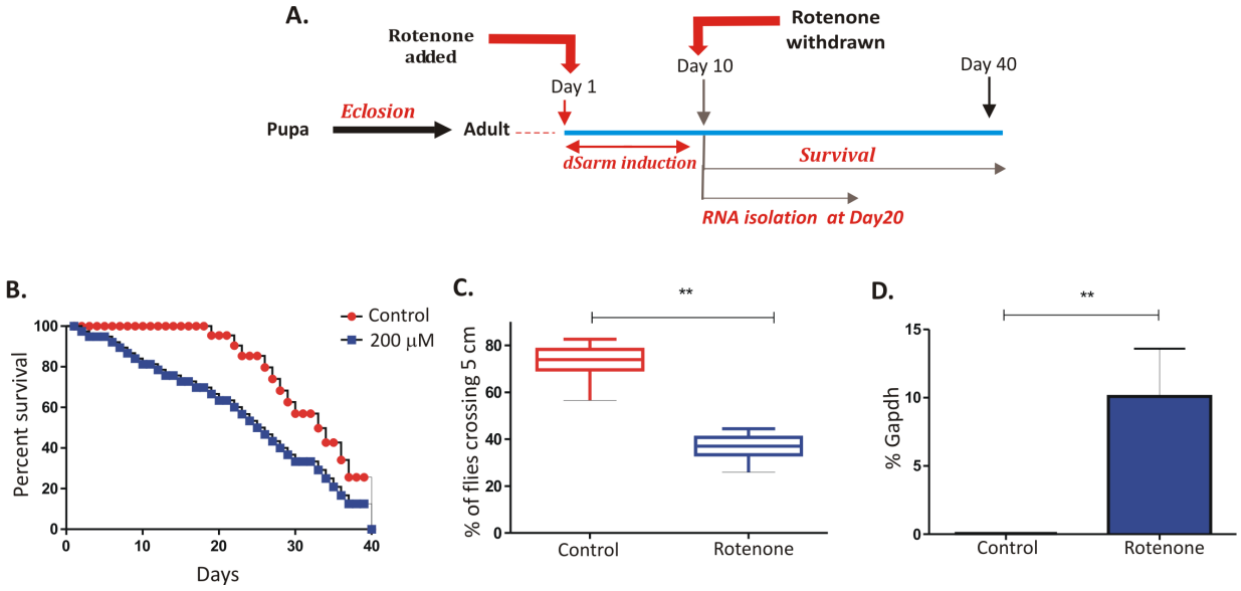


Fig28. *(A) Schematic representation of rotenone treatment of 1-day old flies for 10-days followed by withdrawal and further experiments were performed at the time points indicated. (B) Survival curve of control adult flies and flies exposed for 10-days to 200 µM of rotenone and subsequent transfer of these flies to normal media. Fly viability was scored over a period of 40-days using a minimum of 100 flies per treatment. (C) Negative geotaxis assay of adult flies exposed to rotenone as indicated above for 10-days and experiment performed at day-20 post-exposure. (D) dSarm expression was analysed at day 20 of flies treated above and results compared to age-matched untreated control flies. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to control flies*

5.13. Rotenone induced locomotor deficits is not regulated by increased ROS generation

The pesticide rotenone has been shown to induce neuronal apoptosis via ROS generation. Taking cue from these observations, we exposed w^{1118} flies with rotenone along with the antioxidant N-acetylcysteine (NAC). Interestingly, NAC did not reverse rotenone induced toxicity (Fig. 29A) as well as locomotor deficits (Fig. 29B) in these flies. However, ROS levels were moderately elevated in these flies following 10 days exposure to 200 µM rotenone which was reversed by the addition of NAC (Fig 29C). Hence our data suggest that rotenone induced ROS generation is not the key factor in the DA neuronal loss and *dSarm* induction may act independently of ROS generation.

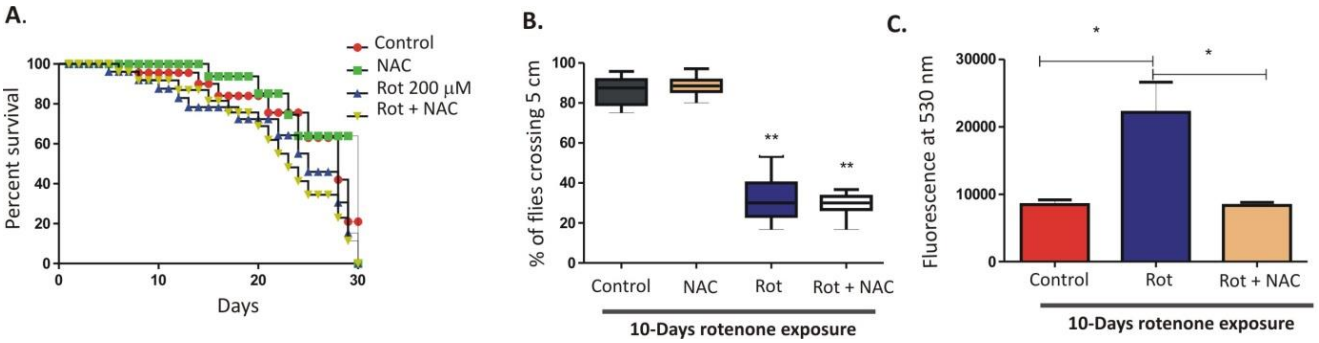


Fig.29. *(A) Survival curve of control adult flies and flies exposed to 200 µM of rotenone in the presence or absence of the ROS scavenger N-acetyl-cysteine or NAC (1 mg/ml). Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. (B) Negative geotaxis assay of the flies at 10-days post-exposure. (C) ROS generation assay in 1-day old flies exposed to 200 µM rotenone for 10-days in the presence or absence of NAC. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.14. Age-dependent increased susceptibility to rotenone was accompanied by heightened inflammatory response

The molecule Sarm1 has been linked to modulate TNFα production to restrict viral infection. Since the brain of rotenone exposed drosophila showed increased *dSarm* expression, we also analyzed the expression of *Eiger* (TNFα homolog in Drosophila) in these flies. Significant increase (P <0.01) in *Eiger* expression was

observed in the brains of younger flies (1-day post eclosion) exposed to 200 µM rotenone (Fig. 30A).

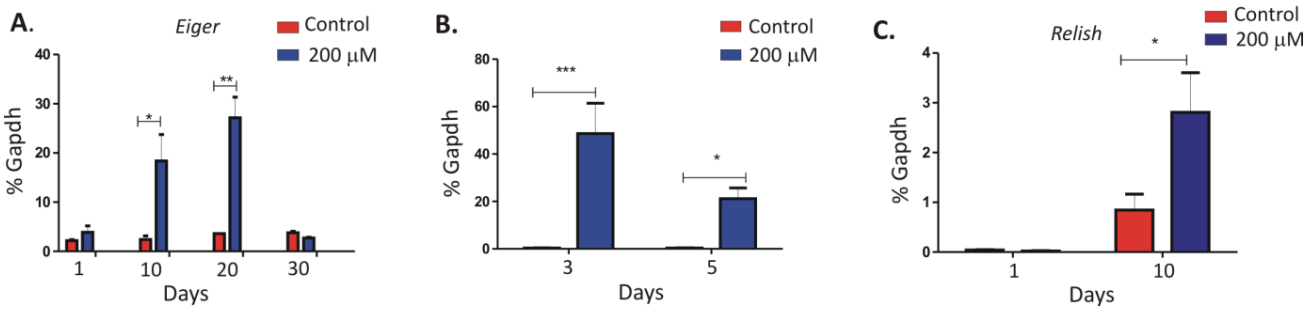


Fig.30. *(A, B) Eiger expression in younger (1-day old) flies exposed to 200 µM of rotenone for 1-day, 10-days, 20-days, and 30-days (a) and aged (20-days old) flies to 200 µM of rotenone for 3-days and 5-days post-treatment (B). (C) Expression of Relish in 1-day old flies exposed to 200 µM of rotenone for 1-day (left panel) and 10-days (right panel) (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control flies*

To correlate the age-dependent susceptibility with increased inflammatory response, 20-day old flies were exposed to rotenone and brains were collected at 3-days and 5-days post exposure. There was a significant increase in *Eiger* expression (Fig. 30B) compared to flies similarly exposed at 1-day post exposure. However, the inflammatory response were significantly reduced in the 5-days treated flies compared to the 3-days post-treatment that correlated with *dSarm* expression in these flies. However, our results showed that the NFκB protein *Relish* was upregulated in the younger flies following exposure to rotenone for 10 days (Fig. 30C) and thus may serve as an upstream activator of the Eiger pathway in the flies following rotenone treatment which needs to be investigated further.

Analysis of other immune response genes (*Upd1*, *Upd2*, *Upd3* and *Wnt*) showed no significant increase in the flies exposed to 200 µM of rotenone in the young as well as aged flies (Fig. 31).

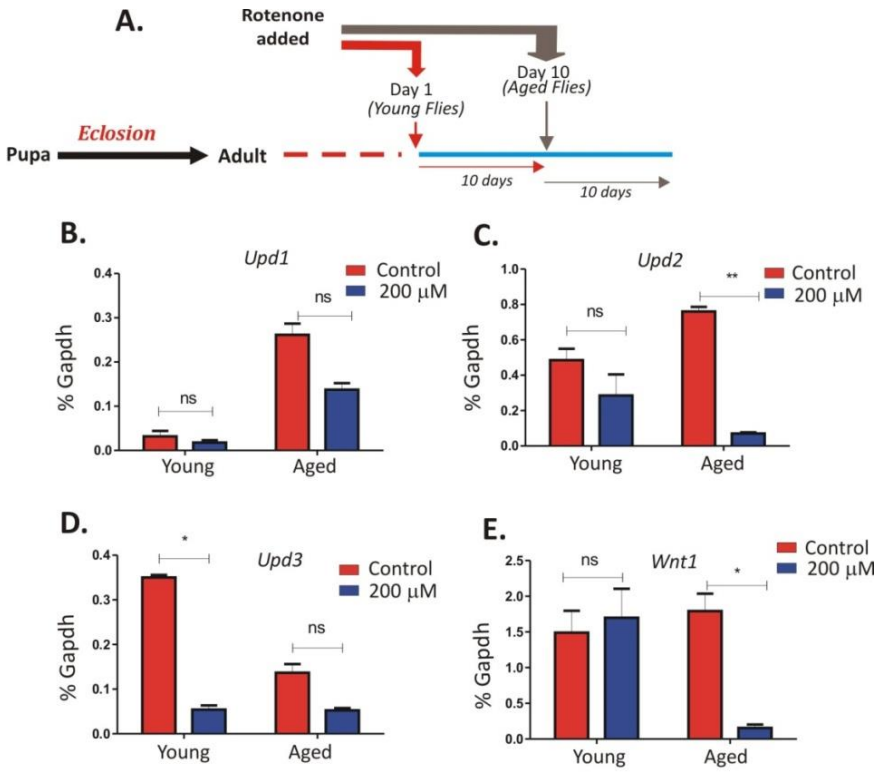


Fig.31. *(A) Schematic representation of experimental setup for rotenone treatment in the young (1-day old) and aged (20-days old) flies. (B-E) Upd1,2,3 and Wnt1 expression in younger (1-day old) flies exposed to 200 µM of rotenone and aged (10-days old) flies to 200 µM of rotenone. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

5.15. The anti-inflammatory molecule Resveratrol reduced *dSarm* expression and rescued rotenone induced locomotor deficits

The polyphenolic compound resveratrol (trans-3,4',5-trihydroxystilbene) found naturally in grapes and nuts, has been shown to be a promising neuroprotective agent acting through the downregulation of the inflammatory responses. To understand whether rotenone induced induction of the inflammatory responses plays a role in the age-associated susceptibility, w^{1118} flies were exposed to 200 µM rotenone at 1-day post eclosion in the presence or absence of resveratrol. Our data indicated that with increasing age, the flies were more susceptible to rotenone induced toxicity in the presence of resveratrol (Fig. 32A). This finding correlates with the idea that mild inflammation has neuroprotective effects and complete abrogation of the inflammatory responses that occurs in these flies with continuous exposure to resveratrol may have detrimental effects in the CNS. As our previous findings suggested that an early induction of *dSarm* may play an important role in the subsequent age-associated susceptibility to rotenone, we exposed the flies to 200 µM rotenone with or without resveratrol (1 µM) at 1-day post eclosion and negative geotaxis assay was performed at 10-days following exposure. A significant reversal ($P < 0.001$) of motor deficits was observed in the presence of resveratrol in the adult flies at 10-days post exposure

where *dSarm* increases significantly as shown previously (Fig. 32B). To further investigate whether resveratrol mediated suppression of the inflammatory responses played an important role in this process, we analyzed the expression of *Eiger* in these flies as early as 1-day post exposure. A significant reversal of the inflammatory responses as seen by the reduction in *Eiger* expression was observed in these flies in the presence of resveratrol that was accompanied by a reduction in *dSarm* expression (Fig. 32C&D respectively) that strongly suggests that the inflammatory responses may act upstream of *dSarm* activation in these flies.

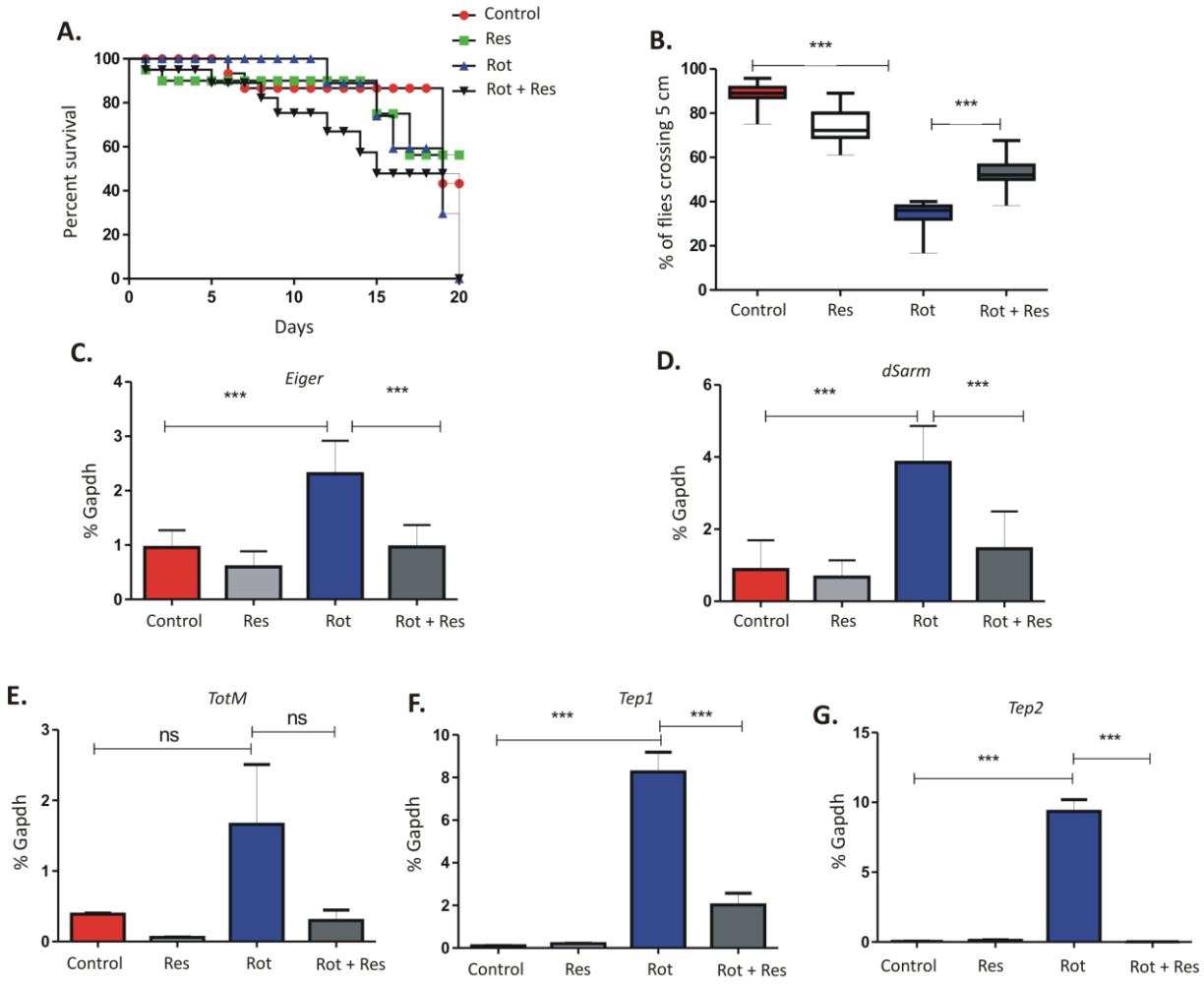


Fig.32. *(A) Survival curve of 1-day old flies exposed to 200 μM of rotenone in the presence or absence of 1 μM of resveratrol. Fly viability was scored over a period of 20-days, using a minimum of 100 flies per treatment. (B) Negative geotaxis assay of the same samples at 10-days post-exposure. (C, D) Real-time PCR analysis of fly heads as treated above. Both Ect4 (C) and Eiger (D) genes were analysed in the 1-day old flies following 1-day post-exposure to rotenone (200 μM) in the presence or absence of resveratrol (1 μM) and results compared to age-matched untreated control flies. E–G Real-time PCR analysis of fly heads as treated above. TotM (E), Tep1 (F), and Tep2 (G) genes were analysed in the 1-day old flies following 1-day post-exposure to rotenone (200 μM) in the presence or absence of resveratrol (1 μM) and results compared to age-matched untreated control flies. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control flies.*

Apart from the anti-inflammatory responses, resveratrol has been shown to induce *Sirt1*, a member of the NAD^+ dependent protein deacetylase family that has important role in aging and life span. Resveratrol has also been shown to regulate AMPK responses in both SIRT1-dependent and independent manner. Our results indicate that resveratrol treatment in flies exposed to rotenone elevates *Sir2* gene (drosophila homolog of mammalian *Sirt1*) expression levels (Fig. 33A) with no effect on *Ampk* expression (Fig. 33B), the mechanism of which needs to be further explored.

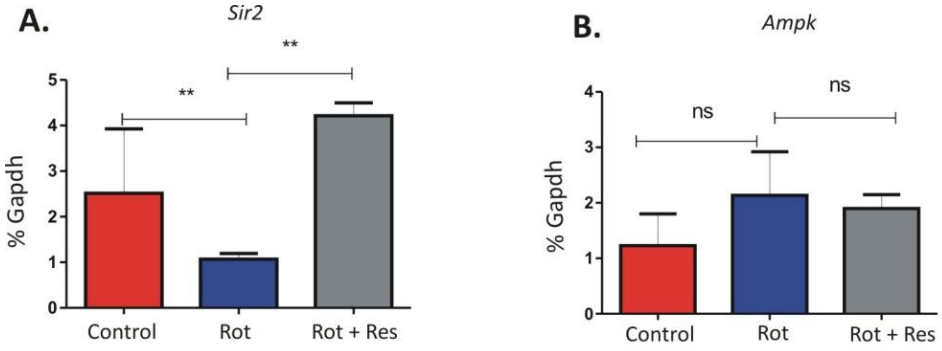


Fig.33. *Real-time PCR analysis of fly heads. Both Sir2 (A) and Ampk (B) genes were analysed in the flies following exposure to rotenone (200 μM) in the presence or absence of resveratrol (1 μM). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample*

5.16. Nicotinamide riboside (NR), rescued rotenone induced loss of survival and associated motor deficits

To study the effect of NR on rotenone exposure, W^{1118} flies were exposed to 200 μM rotenone at 1-day and 10-day post-eclosion in the presence or absence of 1 mM NR for 30-days. Flies showed no reversal in rotenone induced toxicity at the higher dose (200 μM) (Fig. 34 A and C) but there was significant reversal at 100 μM of rotenone in these flies in the presence of NR (Fig. 34B). Interestingly, a significant reversal of rotenone induced locomotor deficits was also observed in these flies (Fig.34D).

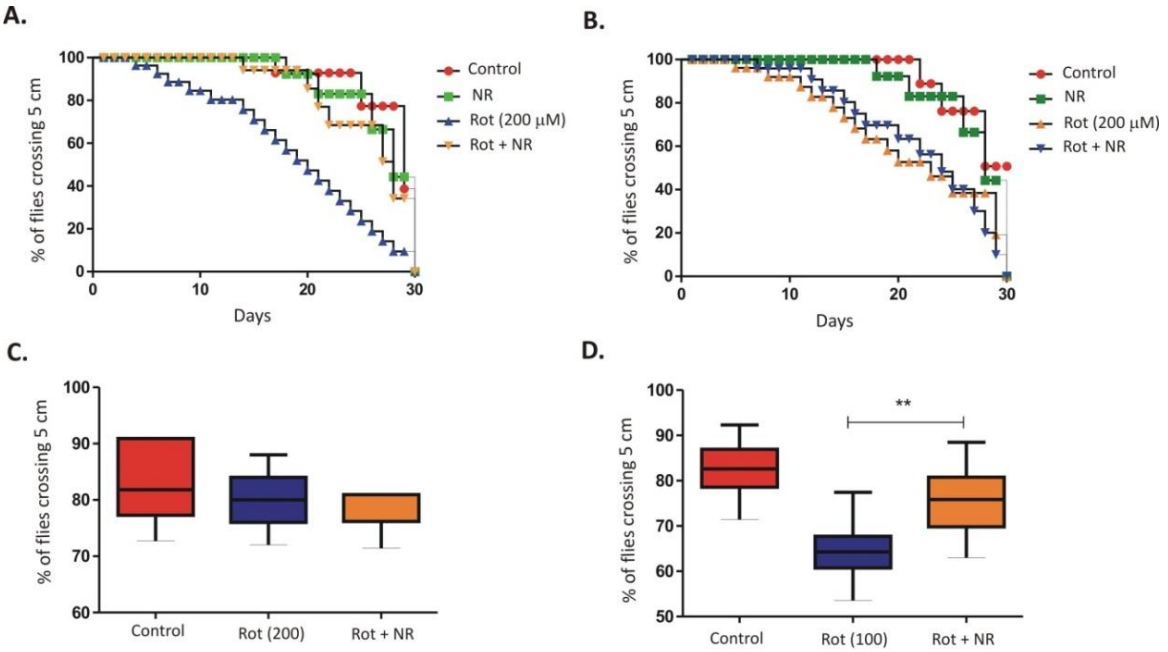


Fig.34. *(A) Survival curve of 1-day (A) and 10-days (B) old flies exposed to 200 μM of rotenone in the presence or absence of 1 mM of NR. Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. (C-D) Negative geotaxis assay of the flies exposed to 200 μM (C) and 100 μM (D) of rotenone in the presence or absence of 1 mM of NR. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

The expression of *dSarm* and the inflammatory genes *Eiger* and *Relish* was also reversed in the 1 and 10-day old flies grown in the presence of NR (Fig.35 A and B).

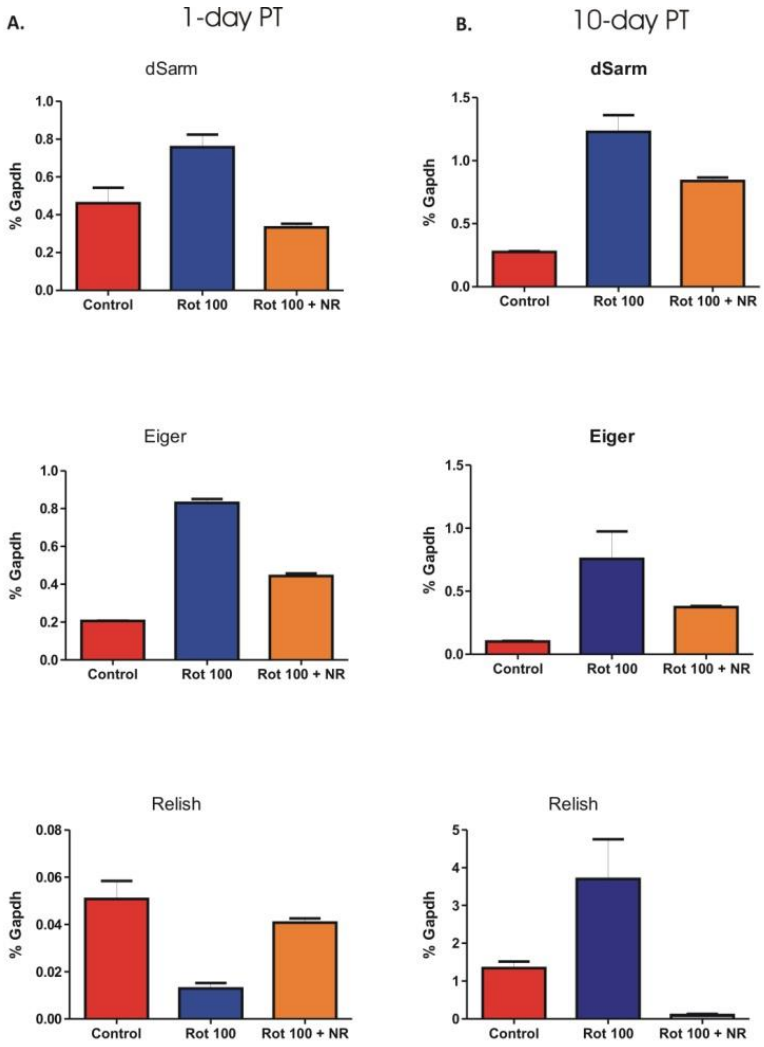


Fig.35. *(A) Real-time PCR analysis of fly heads. dSARM, Eiger and Relish genes were analysed in the 1-day (A) and 10-days (B) old flies following exposure to rotenone (100 μM) in the presence or absence of NR (1 μM). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

To determine the age-related susceptibility to environmental toxins, 20-day old flies were exposed to 100 and 200 μM rotenone with or without rotenone NR. But NR had no effect on increased susceptibility and locomotory deficits of these flies to rotenone exposure (Fig.36 A-B). Interestingly, NR pre-treated flies, exposed to even higher dose (200 μM) of rotenone at 10-day post-eclosion showed a significant reversal in rotenone induced toxicity (Fig.36 C-D).

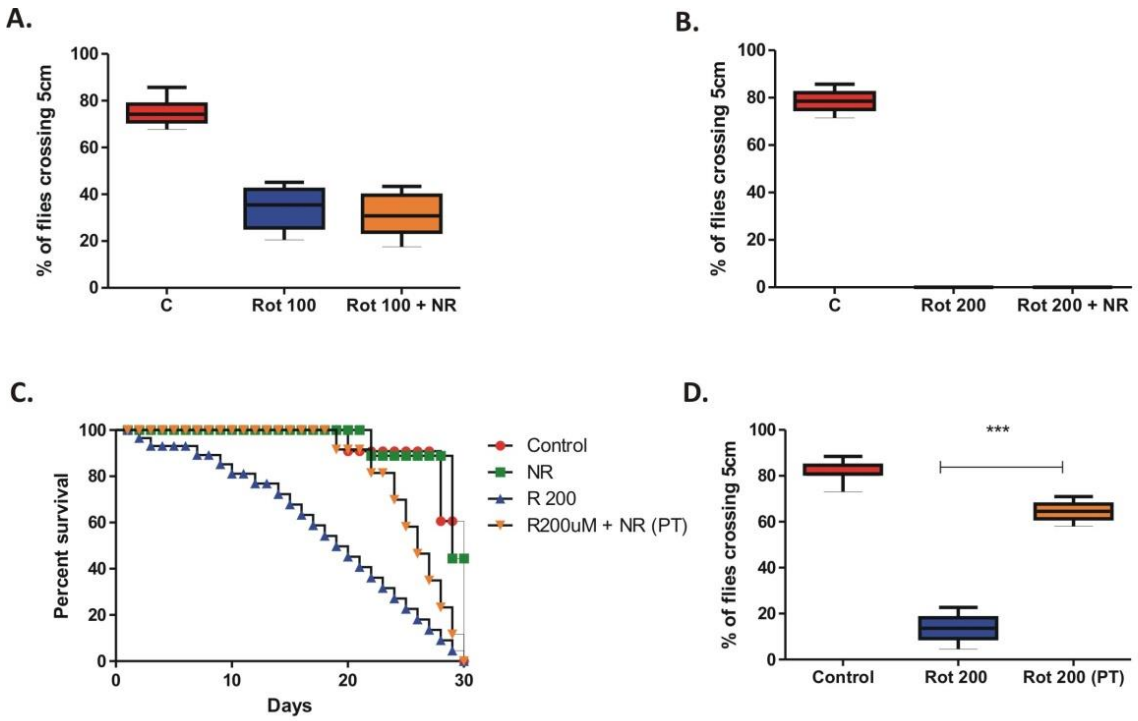


Fig.36. *(A-B) Negative geotaxis assay of the 20-days old flies exposed to 100 μM (A) and 200 μM (B) of rotenone in the presence or absence of 1 mM of NR. (C) Survival curve of NR pre-treated 10-days old flies exposed to 200 μM of rotenone in the presence or absence of 1 mM of NR. Fly viability was scored over a period of 30-days, using a minimum of 100 flies per treatment. (D) Negative geotaxis assay of the same samples. Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Reversal of rotenone induced toxicity in the presence of NR in the aged flies (20-day old) also resulted in reversal of increased induction of *dSarm* as well as *Eiger* (Fig. 37).

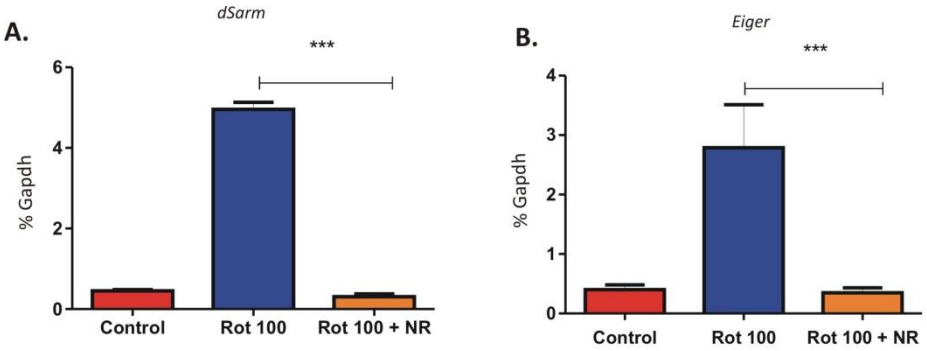


Fig.37. *Real-time PCR analysis of fly heads. dSARM (A) and Eiger (B) genes were analysed in the 20-days old flies following exposure to rotenone (100 μM) in the presence or absence of NR (1 μM). Results are representative of at least three independent experiments. *p < 0.05, **p < 0.01, and ***p<0.001 compared to control sample.*

Conclusion

Our results suggest that rotenone treatment activates the inflammatory responses in the supporting cells of the CNS like microglia resulting in dSarm induction in the neurons. Activation of the dSarm signalling 000network results in rapid loss of dopaminergic neurons of w^{1118} flies eventually leading to motor deficits and loss of survival in the rotenone treated flies. This effect could be partially reversed in the presence of the anti-inflammatory molecule resveratrol indicating that targeting the inflammation induced dSarm pathway could play an important role in age-associated neuronal loss. Alternatively, our data strongly suggests that counteracting NAD+ loss induced by Sarm1 in these flies using the NAD+ precursor NR could reverse rotenone induced neuronal loss and neurotoxicity in an age-dependent manner.

6. Conclusion and impact

In order to understand the mechanism of Sarm1 induced neurodegeneration we used the mitochondrial complex I inhibitor rotenone to correlate mitochondrial dysfunction and Sarm1 regulation. The present study shows that rotenone treatment induced *Sarm1* gene expression and increased mitochondrial localization of the protein. Using knockdown analysis we found Sarm1 is required for rotenone induced death in SH-SY5Y cells. Sarm1 also played a role in the deregulation of ETC complex genes expression that is unique to rotenone treatment but also not the other mitochondrial complex inhibitors. However, although rotenone treatment increased cellular ROS levels, the antioxidant NAC could not reverse rotenone induced cell death which prompted us to explore other cell death mechanisms regulated by Sarm1 like autophagy. Partial reversal of Sarm1 induced neuronal death was observed using NAD precursor (NR), calcium channel blocker (Nimodipine), calcium chelator (EGTA) and an anti-inflammatory molecule (Resveratrol). This suggests that NAD+ levels and calcium may a role in Sarm1 mediated cell death. Our results also suggest that Sarm1 may play an important role to inhibit autophagic flux which induces apoptosis in the later stage of rotenone toxicity.

In vivo studies using aging w^{1118} flies showed that activation of the dSarm signalling network results in rapid loss of dopaminergic neurons of w^{1118} flies that eventually leads to motor deficits and reduced lifespan in the rotenone-treated flies. This effect could be

partially reversed in the presence of the anti-inflammatory molecule resveratrol indicating that targeting the inflammation induced dSarm pathway could play an important role in the age-associated neuronal loss (Fig. 38).

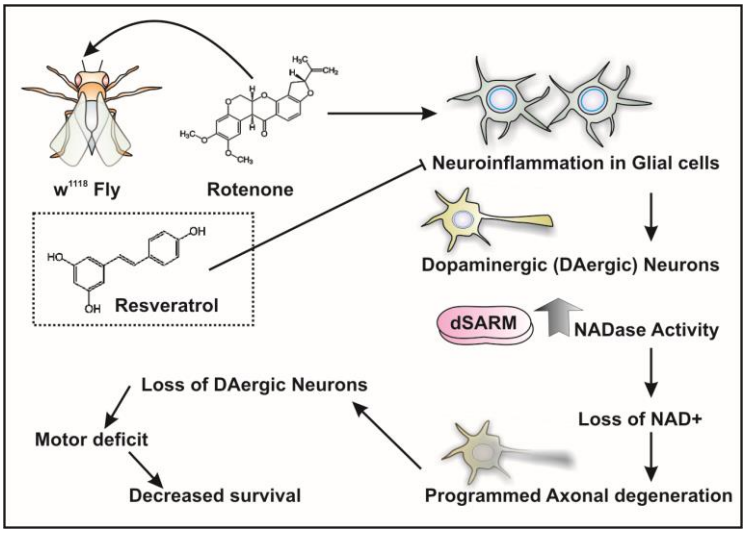


Fig.38. *Proposed model for the study*

The aging model established in flies will help us better understand the increased susceptibility to age-dependent progressive loss of dopaminergic neurons following rotenone exposure. Thus, targeting Sarm1 or the use of anti-inflammatory compounds to reduce its levels could open up cost-effective exciting avenues in the treatment of PD and other age-associated neurodegenerative diseases.

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Table 1: Design of deletion mutants of SARM1

Mutants	Size (bp)
1. ΔNΔARMΔSAM (TIR)	360
2.ΔNΔARMΔSAM2ΔTIR (SAM1)	705
3. ΔNΔARMΔTIR (SAM)	903
4. ΔN (ARM, SAM, TIR)	1440
5. ΔARMΔSAM2ΔTIR (N + SAM1)	327
6. ΔARMΔTIR (N + SAM)	525
7. ΔARMΔSAM1ΔTIR (N + SAM2)	312
8. ΔARMΔSAM (N + TIR)	474

Table 2(a): Sequence of primers used in this study (for SH-SY5Y)

Target genes	Sequences (size)
GAPDH (R)	5'-GCCTTCTCCATGGTGGTGAAGAC-3' (23 mer)
GAPDH (L)	5'-CCCATCACCATCTTCCAGGAGC-3' (22 mer)
Ndufv1 (R)	5'-ACCTTTCAGCCTCCAGTCATGG-3' (22 mer)
Ndufv1 (L)	5'-CACGACAGCACCCAAGAAAACC-3' (22 mer)
Ndufv2 (R)	5'-CCACTCCTTGGCCCTGGTTT-3' (20 mer)
Ndufv2 (L)	5'-GTGGAATGTTTAGGGGCCTGTGT-3' (23 mer)
Ndufs2 (R)	5'-TGCAGGAGCCCGATGTGA-3' (18 mer)
Ndufs2 (L)	5'-ACCCTGAACTTTGGGCCCC-3' (19 mer)
Uqcrfs1 (R)	5'-TCATAGTGTGACCCATGGCA-3' (20 mer)
Uqcrfs1 (L)	5'-TAGATCGAGTAAAGAAACCTGAATGGGTTA-3'(30 mer)
CYC1 (R)	5'-GATGTAGCTGAGGTCAGGGGG-3' (21 mer)
CYC1 (L)	5'-GGGAGATGTTCATGCGGCCA-3' (20 mer)
COX5A (R)	5'-GCAGCATCAATGATTTTGGGCTC-3' (23 mer)
COX5A (L)	5'-GTTTGATGCTCGCTGGGT-3' (18 mer)
COX5B (R)	5'-TGGGGCACCAGCTTGTAATGG-3' (21 mer)
COX5B (L)	5'-GGCTGCATCTGTGAAGAGGACAA-3' (23 mer)
ATP4A (R)	5'-GCGAGGAGTTGTCCACCTTGC-3' (21 mer)
ATP4A (L)	5'-GGCGACCTGGTGGAGATGAAA-3' (21 mer)
ATP4B (R)	5'-GTTGCTGGGGAGGAACTTGAC-3' (21 mer)
ATP4B (L)	5'-AACTGCTCAGGCCTGGCG-3' (18 mer)
ATP12A (R)	5'-CTGGGGCTCAGACTCCCC-3' (18 mer)
ATP12A (L)	5'-AGAGCAGCTGGTGGTGGG-3' (18 mer)
GPX4 (R)	5'-CGTTGGTGACGATGCACACGAA-3' (22 mer)
GPX4 (L)	5'-AAGGACATCGACGGGCACATG-3' (21 mer)
PTGS2 (R)	5'-TCCATCCTTGAAAAGGCGCAGTTTA-3' (25 mer)
PTGS2 (L)	5'-GATGTTTGCATTCTTTGCCCAGCACTTC-3' (28 mer)
SOD2 (R)	5'-GAACTTCAGTGCAGGCTGAAGAGC-3' (24 mer)
SOD2 (L)	5'-GCGCAGATCATGCAGCTGC-3' (19 mer)
TNF α (R)	5'-GGTGTGGGTGAGGAGCAC-3' (18 mer)

TNF α (L)	5’-GAGTGACAAGCCTGTAGCCCA-3’ (21 mer)
HSP70 (R)	5’-CGTGCAGCGGTCGCTATACTC-3’ (21 mer)
HSP70 (L)	5’-GTGGGCATAGACCTGGGCTT-3’ (20 mer)
SARM1 (R)	5’-GCTTGAACATGTGCTCCAAGATGCC-3’ (25 mer)
SARM1 (L)	5’-GAGCAGATCCTGGTGGCTGAGAA-3’ (23 mer)
IL6 (R)	5’-AGTGGCTGTCTGTGTGGGGCG-3’ (21 mer)
IL6 (L)	5’-CCTTCTCCACAAGCGCCTTCGGT-3’ (23 mer)
IL1β (R)	5’-CGCTTTTCCATCTTCTTCTTTGGGTA-3’ (26 mer)
IL1β (L)	5’-GACAAAATACCTGTGGCCTTGGGC-3’ (24 mer)
SIRT1 (R)	5’-AAGTTGTCGGCCAGCGGT-3’ (18 mer)
SIRT1 (L)	5’-TGGGGAAGGAGACAATGGGC-3’ (20 mer)
SIRT3 (R)	5’-AGAATGGGAGTTCAAAAATGGCCT-3’ (24 mer)
SIRT3 (L)	5’-ATCAGCACACCCAGTGGCATT-3’ (21 mer)
SIRT4 (R)	5’-CGATCCAACGGCCTTTTGCTGA-3’ (22 mer)
SIRT4 (L)	5’-AGACGGTCCCACTGTGGG-3’ (18 mer)
SIRT5 (R)	5’-ACATTTCCATTTACTTGGGCCCCG-3’ (24 mer)
SIRT5 (L)	5’-GGACACAGCGCCTCTAGGAGAAAG-3’ (24 mer)
SIRT6 (R)	5’-ACTTGCCCTTGTCCGCGT-3’ (18 mer)
SIRT6 (L)	5’-GAAGCGGCCTCAACAAGGGAAA-3’ (22 mer)
SIRT7 (R)	5’-TGCTCCTCCCGCAACCTC-3’ (18 mer)
SIRT7 (L)	5’-TGCCGTGTGAGGCGGAAG-3’ (18 mer)
AMBRA1 (R)	5’-GCAGGAGAAGGCGGCTTG-3’ (18 mer)
AMBRA1 (L)	5’-GATGTCTGGGGCGTTCTTGTATTCG-3’ (25 mer)
ATG12 (R)	5’-CAGTGGGCGTGCTTTTCTCC-3’ (20 mer)
ATG12 (L)	5’-GACTAGCCGGGAACACCAAGTT-3’ (22 mer)
ATG16L1 (R)	5’-GGCAATCCCGGAGCTCAC-3’ (18 mer)
ATG16L1 (L)	5’-CATGAGCCGGAAGACCGTCC-3’ (20 mer)
ATG4A (R)	5’-CCTGGGACTTGTAGTTCTGCACTG-3’ (24 mer)
ATG4A (L)	5’-GTTTGAGAGCAGTTCCGGGCA-3’ (21 mer)
ATG4B (R)	5’-GTAGCTGCGTCCATCTTCCCAG-3’ (22 mer)
ATG4B (L)	5’-CGGCACACCTATTGGCCC-3’ (18 mer)
ATG4C (R)	5’-CACGCAGACGTTCCGACC-3’ (18 mer)
ATG4C (L)	5’-TCTTACTACGGTGGCCGGG-3’ (19 mer)
ATG4D (R)	5’-ACACTGAGTTCATGGACGCGC-3’ (21 mer)
ATG4D (L)	5’-TAAGATGGCGATGGCTGCGG-3’ (20 mer)
ATG5 (R)	5’-CTGTCATTTTGCAATCCCATCCAGAGTTG-3’ (29 mer)
ATG5 (L)	5’-CTTCTGCACTGTCCATCTAAGGATGC-3’ (26 mer)
ATG9A (R)	5’-ATTCCAGAGGCTCCGCCG-3’ (18 mer)
ATG9A (L)	5’-TGCTTATTGGCCAGCCTGG-3’ (19 mer)
ATG9B (R)	5’-GCCCATTCGGCTCACCATCAG-3’ (21 mer)
ATG9B (L)	5’-ACAGACCTTTGTGGAAGCAACAACA-3’ (25 mer)
BECN1 (R)	5’-GAGCGAGGCCTCCAGAACTAC-3’ (21 mer)
BECN1 (L)	5’-AGCGTCACGTCCGGTCTC-3’ (18 mer)
GABARAP (R)	5’-TCTACTATCACCGGCACCCGG-3’ (21 mer)
GABARAP (L)	5’-AAGAAGAGCATCCGTTCGAGAAGC-3’ (24 mer)
GABARAPL1 (R)	5’-TGAGCCTGCCTGGAGGTAGA-3’ (20 mer)
GABARAPL1 (L)	5’-GCTCTAGCGAAAAGCCGCC-3’ (19 mer)
GABARAPL2 (R)	5’-TGGTCCTCCTTGAACATCCACTTCAT-3’ (26 mer)
GABARAPL2 (L)	5’-TGCCGCCGTCGTTGTTGTT-3’ (19 mer)
IRGM (R)	5’-CTCATAAAGGCAGTGTGGACCCAAA-3’ (25 mer)

IRGM (L)	5’-CTGCTCAGGTCCCCTTCCAT-3’ (20 mer)
MAP1LC3A (R)	5’-GTGTCTGCGGTTTGGGGG-3’ (18 mer)
MAP1LC3A (L)	5’-TCACCGGGCGAGTTACCTC-3’ (19 mer)
MAP1LC3B (R)	5’-CGGCATGGTGCAGGGATC-3’ (18 mer)
MAP1LC3B (L)	5’-TGAGGAGATACAAGGGAAGTGGCT-3’ (24 mer)
RGS19 (R)	5’-CTCAGACCCTCACCACAGCC-3’ (20 mer)
RGS19 (L)	5’-CCCTCCTGCCGTCTGACTTG-3’ (20 mer)
ULK1 (R)	5’-CCCTTGAAGACCACCGCG-3’ (18 mer)
ULK1 (L)	5’-AGAGACCGTGGGCAAGTTCG-3’ (20 mer)
DRAM1 (R)	5’-CCTTTCGCTTTTGGTTTGGACACT-3’ (24 mer)
DRAM1 (L)	5’-TAGGGCCAGAGTGGGAAGG-3’ (19 mer)
ATG3 (R)	5’-CTGTCTGTCCTCGCTTTGCTTCAC-3’ (24 mer)
ATG3 (L)	5’-GCTACGGCAAGAGAGTGAGAAGG-3’ (23 mer)
ATG7 (R)	5’-CTGCAGTTTAGAGAGTCCAGGATCCC-3’ (26 mer)
ATG7 (L)	5’-CAGAGAGAGCTGTGGTTGCCG-3’ (21 mer)
ATG10 (R)	5’-CCAACCAGAAGTGGCCGT-3’ (18 mer)
ATG10 (L)	5’-TGGCGGACCTGACTGAAGG-3’ (19 mer)

Table 2(b):Sequence of primers used in this study (for fly)

Target genes	Sequences (size)
GAPDH (R)	5’-TTGATGTTGGCCGGGTCGC-3’ (19 mer)
GAPDH (L)	5’-GTGGCCGTCAACGATCCCTTC-3’ (21 mer)
ECT4 (R)	5’-ACTTTGCACCGTAATACTGGAGGCC-3’ (25 mer)
ECT4 (L)	5’-AAGGAAGCCCTCTCACTCCGC-3’ (21 mer)
EIGER (R)	5’-TGGTCGCTTTGGCCGGAAAA-3’ (20 mer)
EIGER (L)	5’-GACTGCCGAGACCCTCAAGC-3’ (20 mer)
SIRT1 (R)	5’-TTCGTAATTTTCGAGCGTAGTTTCTGGAA-3’ (29 mer)
SIRT1 (L)	5’-ACATACACACACAGGCGCAGCTA-3’ (23 mer)
TEP1 (R)	5’-CAGAGTAGTCAGGCCCACTT-3’ (20 mer)
TEP1 (L)	5’-TCTGTAAAGCGGGGTGAAGT-3’ (20 mer)
TEP2 (R)	5’-AGCAATTACCTCACCTCGCT-3’ (20 mer)
TEP2 (L)	5’-CCTCATGGGTGGTTACTGGT-3’ (20 mer)
TOTM (R)	5’-GCTGTCGATGTTCCGGTATT-3’ (20 mer)
TOTM (L)	5’-GCCAAGCCTGCACTATGAAT-3’ (20 mer)
UPD1 (R)	5’-AACTGCTATCACTGTTCCTCCCTGA-3’ (25 mer)
UPD1 (L)	5’-GCATCGAAGCACCCGACC-3’ (18 mer)
UPD2 (R)	5’-ATCACTAGCAGCACCTGCCG-3’ (20 mer)
UPD2 (L)	5’-GCCAGCCAACAGAGCCAAA-3’ (19 mer)
UPD3 (R)	5’-CAGGCGAATCAGGCGACTTTG-3’ (21 mer)
UPD3 (L)	5’-TTCGTCCAGCCGCGATATAAAGATAC-3’ (26 mer)
WNT (R)	5’-AGGAGAGTGGTGCCTGGAAC-3’ (20 mer)
WNT (L)	5’-TATTACGAGCACTCTGGCAGCAG-3’ (23 mer)
AMPK (R)	5’-GATGTCAAAGTGGTTGTGGATGTGTC-3’ (26 mer)
AMPK (L)	5’-GCCGCGCTCGAATAGAAATCCA-3’ (22 mer)

F=Forward primer

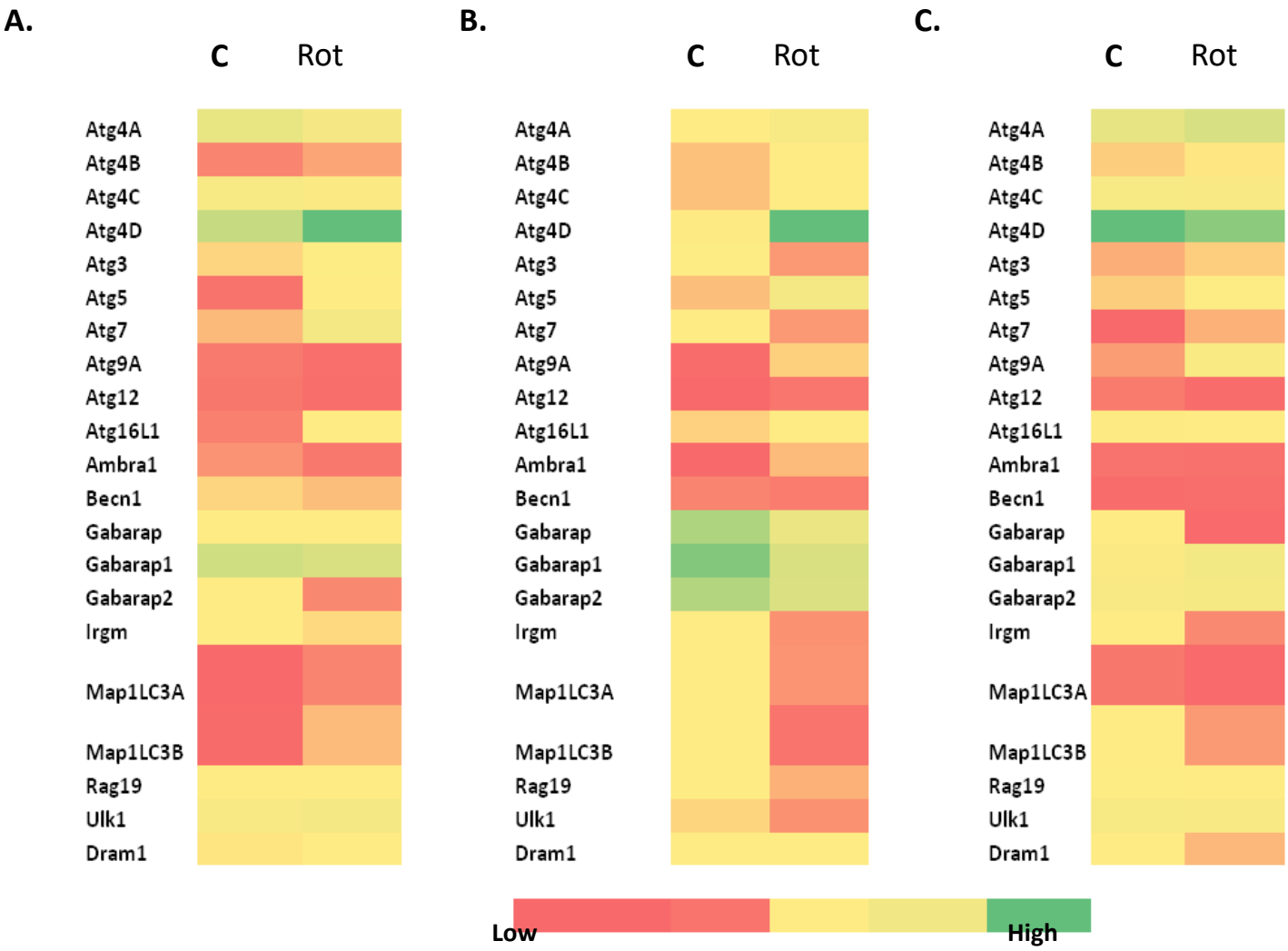
R=Reverse primer

Table 3: List of primary and secondary antibodies used in this study

Protein to be detected	Primary antibody used and its dilution	Time of incubation	Secondary antibody used
β-actin	Anti- β-actin mouse monoclonal antibody (abcam) used at a dilution of 1:1000	*2h **overnight	Goat anti-mouse IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
Caspase-3	Anti- Caspase-3 rabbit polyclonal antibody (abcam) used at a dilution of 1:200	*2h **overnight	Goat anti-rabbit IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
COX4	Anti- COX4 mouse monoclonal antibody (Thermo) used at a dilution of 1:1000	*2h **overnight	Goat anti-mouse IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
FLAG tagged protein	Anti- FLAG mouse monoclonal antibody (Thermo) used at a dilution of 1:1000	*2h **overnight	Goat anti-mouse IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
SARM1	Anti- SARM1 rabbit polyclonal antibody (Cell Signaling) used at a dilution of 1:1000	*2h **overnight	Goat anti-rabbit IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution
Tyrosine hydroxylase	Anti- TH rabbit polyclonal antibody (Merck) used at a dilution of 1:1000	*2h **overnight	Goat anti-rabbit IgG H&L (HRP) preadsorbed (abcam) at 1/5000 dilution

*Blocking with 5% skimmed milk for 30 min at room temperature.

**Incubation with antibody for 2 h at room temperature or overnight at 4 degree C.


A.
C
Rot
Atg4A
Atg4B
Atg4C
Atg4D
Atg3
Atg5
Atg7
Atg9A
Atg12
Atg16L1
Ambra1
Becn1
Gabarap
Gabarap1
Gabarap2
Irgm
Map1LC3A
Map1LC3B
Rag19
Ulk1
Dram1
B.
C
Rot
Atg4A
Atg4B
Atg4C
Atg4D
Atg3
Atg5
Atg7
Atg9A
Atg12
Atg16L1
Ambra1
Becn1
Gabarap
Gabarap1
Gabarap2
Irgm
Map1LC3A
Map1LC3B
Rag19
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Atg16L1
Ambra1
Becn1
Gabarap
Gabarap1
Gabarap2
Irgm
Map1LC3A
Map1LC3B
Rag19
Ulk1
Dram1
Low
High